MBL/WHOI
0 0301 0013073 8

QS 78.6
G 58

MICROSCOPIC HISTOCHEMISTRY

Principles and Practice

By

GEORGE GOMORI, M.D.

THE UNIVERSITY OF CHICAGO PRESS

The original research work reported in this book has been supported by grants from the Douglas Smith Foundation for Medical Research of The University of Chicago and from the Pathology Study Section of the United States Public Health Service.

THE UNIVERSITY OF CHICAGO COMMITTEE
ON PUBLICATIONS IN BIOLOGY AND MEDICINE

EMMET B. BAY • LOWELL T. COGGESHALL
LESTER R. DRAGSTEDT • FRANKLIN C. McLEAN
THOMAS PARK • WILLIAM H. TALIAFERRO

THE UNIVERSITY OF CHICAGO PRESS, CHICAGO 37
Cambridge University Press, London, N.W. 1, England
W. J. Gage & Co., Limited, Toronto 2B, Canada

Copyright 1952 by The University of Chicago. All rights reserved. Published 1952. Composed and printed by THE UNIVERSITY OF CHICAGO PRESS, *Chicago, Illinois, U.S.A.*

No illustration or any part of the text may be reproduced without permission of The University of Chicago Press

TABLE OF CONTENTS

67979

ADDITIONAL REFERENCES

INDEXES

INTRODUCTION

HISTOCHEMISTRY is a borderline field between histology and analytical chemistry or biochemistry. Its subject matter is the identification and localization of chemical substances in the tissues on a cytological scale. In the present book the term will be used in a more restricted sense to include only those methods in which the identifying chemical reaction is observed directly through the microscope, in tissues of which the architecture is not grossly altered. This definition will eliminate at once two other important ways of approach: (1) those in which certain morphological structures (nuclei, mitochondria, etc.) are first separated by physical means, such as differential solubility or centrifugation, and are then analyzed chemically and (2) the ingenious statistical methods developed by Linderstrøm-Lang[1] and his school. "Cytochemistry" is often used as a synonym; however, it should be reserved for the study of the chemical organization of the cell in general.[2]

Histochemistry is a young science, although a few histochemical reactions have been known for over seventy years (iodine reaction for starch;[3] Prussian blue reaction for iron[4]). Actually, probably most of the staining techniques described since the earliest days are based on some chemical or physicochemical interaction between dye and tissue; however, they cannot be called "histochemical" for two reasons: (1) the underlying chemical reactions are not understood, and (2) their results, valuable as they may be for the differentiation of various morphological structures, do not convey any information about their chemical constitution.

1. Linderstrøm-Lang, K.: Bull. New York Acad. Med., **15**:719, 1939.
2. Dick, A. T.: Australian Chem. Inst. J. & Proc., **15**:294, 1948.
3. Caventou, J. B.: Ann. de chim. et phys., **31**:337, 1826.
4. Perls, M.: Virchows Arch. f. path. Anat., **39**:42, 1867.

Although there had been a few surveys of histochemical methods published between 1920 and 1930, it was Lison[5] in 1936 who made the first real effort to organize all existing knowledge into the science of histochemistry. By introducing a systematically critical attitude into the new discipline and by establishing the criteria of validity, he gave histochemistry the standing of a science and succeeded in clearing away much pseudo-scientific rubbish from the path of advancement. He fully deserves to be called the "founder of histochemistry."

Since the late 1930's histochemistry has undergone a remarkably rapid development. A large number of important new methods have been devised, and older methods have been subjected to critical analysis. Histochemical methods have become a routine in many laboratories of histology and pathology, and the number of papers reporting results obtained with their use has been increasing by leaps and bounds. The rate of development is best reflected in the number of histochemical review articles, chapters, and books printed since 1940. Not fewer than thirteen such comprehensive works[2,6] were published between 1941 and 1951, while the corresponding number for the period between 1912 and 1940 is only six,[5,7] exclusive of reviews on microinciner-

5. Lison, L.: Histochimie animale (Paris: Gauthier-Villars, 1936).

6. Gersh, I.: Physiol. Rev., **21**:242, 1941; Glick, D.: Ann. Rev. Biochem., **13**:705, 1944; Gomori, G.: J. Mt. Sinai Hosp., **11**:317, 1945; Dempsey, E. W., and Wislocki, G. B.: Physiol. Rev., **26**:1, 1946; Glick, D.: Techniques of histo- and cytochemistry (New York and London: Interscience Publishers, 1949); Glick, D.: Adv. Enzymol., **9**:585, 1949; Holter, H.: Oesterreich. Chem. Ztschr., **50**:204, 1949; Bradfield, J. R. G.: Biol. Rev. Cambridge Phil. Soc., **25**:113, 1950; Dounce, A. L.: Cytochemical foundations of enzyme chemistry, in Sumner and Myrbäck, The enzymes (New York: Academic Press, 1950); Gomori, G.: Microchemical tests for certain substances other than fats and chromatin, in Bolles Lee, The microtomist's vademecum (11th ed.; London: J. A. Churchill, 1950); Glick, D., Engström, A., and Malmström, B. G.: Science, **114**:253, 1951; Gomori, G.: Histochemical staining methods, in Methods of medical research (Chicago: Year Book Publishers, 1951); and Pearse, A. G. Everson: J. Clin. Path., **4**:1, 1951.

7. Macallum, A. B.: Die Methoden der biologischen Mikrochemie, in Abderhalden's Handb. d. biol. Arbeitsmeth., **V-2**, 1099 (Berlin and Vienna:

ation. There is every indication that histochemistry is emerging now as an independent discipline with its own theoretical background, methods, and special problems, just as was the case with biochemistry shortly after the turn of the century.

Urban & Schwarzenberg, 1912); Prenant, A.: Rev. gén. d. sc., **32**:581, 1921; Parat, M.: Biol. Rev., **2**:285, 1927; Patzelt, V.: Animale Histochemie, in Klein and Strabinger's Fortschritte der Microchemie (Leipzig and Vienna: F. Deuticke, 1928); and Klein, G.: Praktikum der Histochemie (Berlin: J. Springer, 1929).

PART I

HISTOCHEMICAL METHODS IN GENERAL

CHAPTER I

THE NATURE OF THE PROCESSES OF IDENTIFICATION IN HISTOCHEMISTRY

Most of the methods used in histochemistry have been borrowed from other fields of chemistry and partly modified for the purpose of application to histological preparations. The methods can be divided into several classes, according to the nature of the procedures and phenomena utilized.

1. *Chemical.*—Some of the reactions of this class are the same as those used in analytical chemistry or biochemistry (for Fe^{+++}, Cl^{-}, enzymes, etc.). However, modifications of the original technique are often necessary; e.g., determination of melting point cannot be used in histochemistry; colorless or only slightly colored precipitates are transformed, whenever possible, into intensely colored ones for better visibility.

2. *Semichemical.*—Reactions in this class are more or less specific for certain chemically definable substances, but the nature of the reaction is poorly understood (Best's carmine for glycogen; mucicarmine for mucin).

Mere staining with dyes of acid or basic character, although it does reveal something about the acid- or base-combining properties of the substance stained (basic dyes for nucleic acids; acid dyes for globins and histones), cannot be called a histochemical method in any true sense of the word.

3. *Physical.*—This class can be subdivided into several subclasses.

a) *Staining of fat.*—This is a purely physical phenomenon of solubility in oil, without any chemical reactions taking place.

b) *Fluorescence.*—A number of substances show various kinds of fluorescence under ultraviolet light. Some of the most typical are the fading green-blue fluorescence of vitamin A, the golden-yellow fluorescence of enterochromaffin granules, and the brown one of ceroid. Great care should be exercised in the evaluation of the results, and, if possible, the emitted fluorescent light should be analyzed spectroscopically.

Staining with fluorescent dyes is not a histochemical method, any more than staining with other, nonfluorescent, dyes.

c) *Ultraviolet spectrography.*—This was developed by Caspersson[1] and his school. A number of compounds, such as nucleic acids and some amino acids, have characteristic absorption spectra in the ultraviolet and can be identified by them accurately and in a quantitative fashion. A fairly complicated and expensive equipment is required, consisting of a suitable ultraviolet light source, a quartz optical system for the microscope, and a highly sensitive microphotometer. Areas of the size of 1 μ^2 can be used for analysis.

d) *X-ray spectrography* (*Engström*).[2]—This is an even more complicated procedure, with a difficult theoretical background. However, it permits a highly accurate quantitation of almost any element (but not of compounds) in very small areas.

e) *Spark spectrography.*[3]—The emission spectrum of a part of a tissue section vaporized in a spark gap is analyzed.

f) *Tracer techniques.*[4]—These techniques utilize the emis-

1. Caspersson, T.: Arch. f. Physiol., Vol. **73,** Suppl. 8, 1936, and J. Roy. Micr. Soc., **60**:8, 1940.

2. Engström, A.: Acta radiol., Suppl. 63, 1946.

3. Gerlach, W., and Gerlach, W.: Die chemische Emissionsspektralanalyse. II. Anwendung in Medizin, Chemie und Mineralogie (Leipzig: Voss, 1933); Policard, A., and Morel, A.: Bull. d'histol. appliq. à la physiol., **9**:57, 1932; Policard, A.: Protoplasma, **19**:602, 1933; and Scott, G. H., and Williams, P. S.: Proc. Soc. Exper. Biol. & Med., **32**:505, 1934.

4. Hamilton, J. G.: J. Appl. Physiol., **12**:440, 1941; Simpson, W. L.: Radioactive isotopes, in Cowdry, Laboratory technique in biology and medi-

sion of energy-rich radiation by suitable isotopes. The tissue sections are mounted directly on a photographic emulsion, which, after a certain length of exposure, is developed. The unreacted silver halide is removed by hypo (Na thiosulfate), and the section can be stained by one of the conventional histological staining methods. Radioactive substances are revealed by local blackening of the emulsion.

4. *Physicochemical.*—In this class belong methods which attempt to obtain information about the dissociation constants of protein substances by staining them with dyes buffered at various pH levels. As will be shown, the validity of inferences drawn from the results of this method is open to doubt.

5. *Microincineration.*[5]—This technique cannot be fitted easily into any of the previously mentioned classes. Considerable experience is required for the evaluation of spodograms; however, it seems that at least Fe and Si can be relatively easily recognized in the ash.

The present book will be concerned only with techniques belonging in classes 1, 2, 3*a*, 3*b*, and 4. They require little or nothing in excess of the apparatus found in any reasonably well-equipped laboratory of histology.

---

cine (2d ed.; Baltimore: Williams & Wilkins, 1948); and Kurbatov, J. D., and Pool, M. L.: Chem. Rev., **32**:231, 1943.

5. Policard, A., and Okkels, H.: Anat. Rec., **44**:349, 1930; Scott, G. H.: Protoplasma, **20**:133, 1933, and Am. J. Anat., **53**:243, 1933; Policard, A.: Compt. rend. Assoc. anat., **29**:463, 1934; Gage, S. H.: Stain Technol., **13**:25, 1938; and Scott, G. H.: Biol. Symp., **10**:277, 1943.

CHAPTER II

THE SPECIAL FEATURES OF HISTOCHEMICAL METHODS

The usual requirements of a satisfactory reaction in analytical chemistry are specificity and sensitivity. In histochemistry, since one of the main objects is accurate localization, two more conditions must be fulfilled.

First of all, the chemical substances to be identified must be immobilized at the sites they have occupied in the living tissue. This is no problem as long as substances such as calcium phosphate, hemosiderin, lipids, etc., insoluble in well-chosen fixatives, have to be demonstrated. It is a relatively minor problem in the case of large and poorly diffusible molecules, like proteins and glycogen. These are, as a rule, precipitated by the fixative or emmeshed inextricably in a spongework of other co-precipitated substances before any gross displacement can take place. It must be remarked, however, that minor shifts on a cytological scale are not necessarily prevented. As the fixative penetrates into the interior of the tissue, the advancing front of a high-concentration gradient (especially when a fixative which acts partly by dehydration, such as alcohol or acetone, is used) may push certain substances ahead of itself until they are stopped by an impermeable barrier, such as a cell membrane. In this way artifacts of the type of the well-known "glycogen flight" may be produced. They are usually most marked near the surface of the tissue block, where the tissue is hit by a sudden high concentration of the fixative.

In the case of easily soluble and highly diffusible substances (ions, sugars, ascorbic acid, urea, etc.), the regular methods of fixation cannot effect an immobilization, even

if the fixative is applied in the form of a gas (formaldehyde vapor) or if it contains a specific precipitant for the compound investigated. The spontaneous diffusion of small molecules, together with the violent drift of solutes, caused by the difference in osmotic pressure (global or specific) between tissue fluids and the fixative, will result in a more or less marked distortion in the pattern of distribution of these molecules within a short time. Applying the fixative by means of vascular perfusion may eliminate gross displacement but not the intracellular shift of solutes.

Such a displacement of highly diffusible substances can be prevented by the use of the freezing-drying technique, originally described by Altmann[1] and perfected by Gersh.[2] For a detailed description of the apparatus and its use the reader is referred to the bibliography;[3]—only the principles of the procedure will be given here.

Small pieces of fresh tissue are dropped into liquid air (or, even better, into isopentane cooled by liquid air; temperature lower than −150° C.) where they are frozen solid almost instantly. Subsequently, they are dehydrated *in vacuo* at a temperature around −30° C. This dehydration may take from a few hours to weeks, depending on the temperature, the efficiency of the vacuum, and the size of the tissue, etc. The dehydrated tissue is then embedded in paraffin. The important point is that there is no liquid phase present at any stage of the procedure; therefore, diffusion of solutes cannot take place. The sections may be floated directly on the reagent (for instance, an alcoholic solution of silver nitrate

1. Altmann, R.: Die Elementarorganismen und ihre Beziehungen zu den Zellen (Leipzig: Veit & Co., 1890).

2. Gersh, I.: Anat. Rec., **53**:309, 1932, and Bull. Internat. A. M. Mus., **28**:179, 1948.

3. Hoerr, N. L.: Anat. Rec., **65**:293, 1936; Hoerr, N. L., and Scott, G. H.: Frozen-dehydration method for histologic fixation, in Glasser, Medical physics (Chicago: Year Book Publishers, 1944); Simpson, W. L.: Anat. Rec., **80**:173, 1941; Packer, D. M., and Scott, G. H.: Bull. Internat. A. M. Mus., **22**:85, 1942; and Stowell, R. E.: Stain Technol., **26**:105, 1951.

for Cl^-) or mounted on slides and processed after the removal of paraffin.

Obviously, the freezing-drying method offers great advantages. Diffusion artifacts and shrinkage are largely eliminated; enzymes are very well preserved. However, it also has several disadvantages. The equipment required is bulky and rather expensive; only small tissue fragments can be used if distortion by ice crystals is not to occur; sections of the embedded material are not easy to handle because they are quite sensitive to water. It should be added that, if frozen-dried sections are run down through xylene and alcohols to water in the same way that regular sections are, most of the proteins will not be fixed and will remain soluble. Unless the slides are coated with collodion, a considerable loss of protein substances and glycogen is liable to occur on hydration. A short (2–3 minutes) bath in 70–80 per cent alcohol between the second 95 per cent alcohol and water may be employed to make proteins insoluble.

In spite of its disadvantages, the freezing-drying method is a "must" in certain types of histochemical research, and its application has yielded most valuable information regarding the localization of mobile ions in the tissues.

The second special condition for histochemical localization is that the chemical reaction or physical means utilized for identification should possess certain features which can be enumerated as follows:

1. It must be applicable to reactions *in situ*. Therefore, reactions taking place in solution only (e.g., the Carr-Price[4] reaction for vitamin A; the Kober[5] reaction for estrogens, etc.) are unsuitable.

2. It must preserve tissue structure. Methods utilizing concentrated H_2SO_4 or KOH can have, at best, a very limited value in localization because tissue structure is badly damaged by these reagents.

4. Carr, F. H., and Price, E. A.: Biochem. J., **20**:497, 1926.

5. Kober, S.: Biochem. Ztschr., **239**:209, 1931.

3. The end result produced must be reasonably stable. Soluble or fleeting colors, such as that of the rhodanate reaction[6] for Fe^{+++} or the change in the shade of indicators caused by acid liberated enzymatically from esters, do not localize with sufficient accuracy. A reaction to be used in histochemistry must produce highly insoluble and, preferably, intensely colored precipitates.

4. When a reaction produces a crystalline precipitate, the size of the crystals must be small enough to permit cytological localization. This condition is not fulfilled in the gypsum reaction for Ca[7] or in the digitonine reaction for cholesterol.[8]

5. In the case of soluble substances, the reaction must be prompt, of a speed not much inferior to that of ionic reactions. Slow reactions are unusable. To give an extreme example, some otherwise excellent reactions for glucose (osazone formation; the reduction of Benedict's solution) are entirely unsuitable for histochemical purposes. Glucose would diffuse far from its original site before either of these reactions was completed.

Failure to recognize these simple principles has resulted in a number of publications reporting the histochemical localization of substances for which suitable reactions are not known.[9]

6. Schmelzer, W.: Ztschr. f. wissensch. Mikr., **50**:99, 1933.

7. Schujeninoff, S.: Ztschr. f. Heilk., **18**:79, 1897.

8. Leulier, A., and Noel, R.: Bull. d'histol. appliq. à la physiol., **3**:316, 1926.

9. Seeger, P. G.: Arch. f. exper. Zellforsch., **21**:308, 1938; Ztschr. f. mikr.-anat. Forsch., **48**:181, 639, 1940, and **53**:65, 1943.

CHAPTER III

THE HISTOCHEMICAL ROUTINE

The first few steps in handling tissues for histochemical investigations are essentially the same as those used in histology, except for minor differences due to chemical considerations.

In a few cases fixation is not permissible. Certain sensitive enzymes, especially oxidative, are badly damaged by all known fixatives. In such cases the tissues must be used fresh, in an unfixed condition, either as smears or as frozen sections. Such tissues are not easy to handle; they are extremely fragile and are readily cytolyzed by many reagents. Furthermore, a number of proteins will remain soluble and diffuse from their original sites unless the proper precautions are taken. The only way to keep proteins insoluble but undenatured is to use strong (half-saturated or better) salt solutions [e.g., $(NH_4)_2SO_4$; NaCl; Na acetate] throughout the histochemical procedure, up to the last step, when the final precipitate is produced. In some instances, when the enzyme is resistant to drying out and to moderate heat, the freezing-drying method may be the answer to the problem.

However, in most cases fixation is possible and preferable. Tissues should be fixed promptly, although a few hours' delay, especially if the specimen is refrigerated, seldom causes noticeable changes.

The choice of the right fixative is important. Fixatives giving good cytological detail and a minimum of artifacts should be preferred whenever possible. Often, however, a compromise is necessary; one may have to sacrifice cytological excellence to the preservation of the chemical substance investigated. This applies especially to the preservation of

enzymes. The correct fixative or fixatives will be specified in the description of the individual techniques.

The fixed tissue is either cut on the freezing microtome or embedded in paraffin or celloidin. Formalin-fixed tissues usually give excellent frozen sections, while, after alcohol or especially acetone fixation, frozen sections are very delicate and easily break to pieces. Occasionally, especially when an enzyme is not too sensitive to a relatively short fixation but is sensitive to a long exposure to dehydrating agents and/or heat, a sort of semi-embedding may be carried out as follows: thin slices of the tissue are dehydrated by several changes of absolute alcohol or acetone, a few hours each; subsequently they are transferred to a mixture of equal parts of alcohol and ether for 2 hours, followed by about 4 per cent celloidin in alcohol-ether (Collodion, U.S.P.) for 12–24 hours, hardened in 70 per cent alcohol for a few hours, and finally transferred to water. The entire procedure is preferably carried out at icebox temperature. Tissue blocks infiltrated with thin celloidin by this method can be cut on the freezing microtome; the sections have an excellent consistency and no tendency to break up.

In most cases paraffin or celloidin embedding is possible, and both techniques have their advantages and disadvantages. Celloidin embedding does not require the application of heat, and this may be an important advantage when dealing with heat-sensitive enzymes. However, dilute alcohol, used in the storage of celloidin blocks, is very detrimental to enzymes, and most of them will be destroyed by it in a short time; therefore, it is imperative to cut and process the blocks promptly.

Paraffin embedding usually causes considerable inactivation of enzymes, although in a completely anhydrous state many proteins will resist the denaturing effect of heat remarkably well. However, even traces of water left in the tissue will considerably accelerate the rate of inactivation by heat. That is why thorough dehydration of the tissue is so

important. It is always advisable to avoid excessive heat; the temperature of the paraffin oven should not exceed 56°–58°C. Even at this temperature tissues should not be exposed to heat longer than absolutely necessary. The vacuum technique, to be described in the section on enzymes, will cut down considerably the time required for embedding.

Paraffin sections should be floated on lukewarm water and attached to the slide either without any adhesive or with P. Mayer's egg-white–glycerol mixture. After complete drying, it is advisable to place the slides in the paraffin oven for a few minutes until they melt. The melted paraffin forms an excellent coating on the surface and protects the tissue from the injurious effect of atmospheric oxygen and moisture.

The sections are dewaxed in xylene and carried through alcohols (absolute and 95 per cent) to water as usual. Often it is advisable to protect the tissue with a thin layer of collodion. This is done by flooding the slide after the last alcohol with a dilute (about ½ per cent) solution of celloidin in alcohol-ether, shaking off the excess and hardening the membrane in 80–95 per cent alcohol. The collodion membrane serves two purposes: (1) it prevents diffusion of large molecules not made insoluble by fixation, and (2) it facilitates the removal of nonspecific surface precipitates, a by-product of some histochemical reactions. These precipitates will settle on the surface and can be washed off by dissolving the membrane in alcohol-ether or acetone.

CHAPTER IV

CONTROLS TO PROVE VALIDITY OF TECHNIQUE

In a number of cases the reaction used is so highly specific that no control is necessary; a positive reaction indicates the presence of the substance searched for with absolute certainty (for instance, the Prussian blue reaction for Fe^{+++}). In other cases, however, "blanks" must be run, as in analytical chemistry or biochemistry, to avoid confusing the genuine reaction with other similar reactions of a nonspecific nature. These blank runs are especially important in the identification of enzymes.

The two main methods for the verification of the specificity of enzymatic reactions are (1) the omission of essential ingredients (e.g., the substrate or Ca ions in the technique for alkaline phosphatase) and (2) the use of inactivators or inhibitors, such as excessive heat, strong acids, oxidants, fluoride, eserine, etc., depending on the nature of the enzyme investigated. Whatever reaction persists after such treatment cannot be due to enzymatic activity.

One of the difficulties of identification in histochemistry is the impracticability of applying reactions to purified substances. The compounds investigated almost invariably occur in association with, and often adsorbed on, other compounds. The presence of these may profoundly modify the typical reactions, solubility, color, and other properties of the compound investigated as listed in textbooks of analytical chemistry. In addition, fixatives may cause such significant changes in the reactive groups as to make them unrecognizable by the accepted identifying reactions. These are the main reasons why test-tube reactions, even if they otherwise

meet the standards previously mentioned, may give rise to misleading results when applied to tissues.

The applicability of procedures of analytic chemistry to histochemical research can be tested by model experiments. Such experiments attempt to carry out the identifying reactions under conditions more or less similar to those prevailing in tissue sections. The first, rather primitive, model experiment is credited to Altmann,[1] who investigated the differential staining reactions of various fatty substances imbibed by a piece of tissue paper. In more accurate experiments the substance in question is dissolved or finely dispersed in agar, gelatin, or some similar substance. The suspension can be smeared on slides or allowed to gel, fixed and embedded like any tissue block. Another clever technique has been devised by Coujard,[2] permitting the comparison of a large number of substances on a single slide. The substances to be tested are dissolved in serum, dilute gelatin, or egg-white or some other freely flowing protein solution, and marks are made with a clean steel pen on a carefully cleaned slide, using the solutions as ink. The use of different symbols as marks for the different substances (e.g., abbreviations of their names, chemical formulas) will facilitate prompt and easy recognition of the marks. As soon as the slides are dry, they can be processed as if they were smears. Coujard's method assures the chemical comparison of many different test substances under strictly identical conditions.

Model experiments find a number of applications in histochemistry, of which a few will be mentioned.

1. *Determining the chemical specificity of methods.*—Examples of this will be mentioned in the sections on nucleic acids, lipids, and phenolic substances. Each simple histological staining method with no known chemical background

1. Altmann, R.: Die Elementarorganismen und ihre Beziehungen zu den Zellen (Leipzig: Veit & Co., 1890).

2. Coujard, R.: Bull. d'histol. appliq. à la physiol., **20**:161, 1943.

can be tested. For instance, it can be shown that the staining of beta cell granules in the pancreatic islets by chrome hematoxylin[3] is not due to their insulin content, since marks made with commercial insulin and fixed in Bouin's fluid just like a piece of pancreas do not stain.

2. *Studying the effects of fixation and embedding.*—Model slides (carrying, e.g., marks made with enzyme solutions) can be treated with any combination of fixatives, dehydrating and clearing agents, hot paraffin, etc., and the timing can be varied within wide limits. The effect of these procedures can be judged not only qualitatively but, to a certain extent, even quantitatively (see next chapter) by the outcome of the reaction. Such simple experiments may supplement or even replace those laborious studies in which tissue blocks are used and enzymatic activity, after various treatments, is determined chemically, by test-tube methods.

3. *Quantitation of histochemical methods.*—The important problem of quantitation in histochemistry and the role of model experiments in it will be discussed in the next chapter.

3. Gomori, G.: Am. J. Path., **15**:497, 1939.

CHAPTER V

QUANTITATION IN HISTOCHEMISTRY

The greatest advantage of biochemical over histochemical methods is the far superior ability of the former to quantitate results. However, as will be shown, a modest degree of quantitation can be achieved also by purely histochemical techniques.

Absorption colorimentry is the main tool of quantitation in analytical chemistry, biochemistry, and histochemistry. However, conditions prevailing in microscopic sections only rarely permit the theoretically correct application of colorimetry.

One of the fundamental principles of absorption colorimetry is that the distribution of the absorbing material in the sample must be uniform. If this condition is fulfilled, quantitative evaluation of photometric readings obtained through the microscope is an entirely correct procedure, and it has given much valuable information especially in the hands of Caspersson[1] and his school and a few others.[2] It should be remarked, however, that areas sufficiently uniform in optical density are, as a rule, very small, usually occupying only a minute fraction of an oil-immersion field. The minimum diameter required for a measurement is about four times that of the wave length of the light used.

Irregular distribution of the absorbing material will lead

1. Caspersson, T.: Arch. f. Physiol., Vol. **73**, Suppl. 8, 1936, and J. Roy. Micr. Soc., **60**:8, 1940.

2. Gersh, I., and Baker, R. F.: J. Cell. & Comp. Physiol., **21**:213, 1943; Stowell, R. E.: J. Nat. Cancer Inst., **3**:11, 1942, and Anat. Rec., **91**:301, 1945; Ris, H., and Mirsky, A. E.: J. Gen. Physiol., **33**:125, 1949; Swift, H. H.: Physiol. Zoöl., **23**:169, 1950, and Proc. Nat. Acad. Sc., **36**:643, 1950; and Hoover, C. R., and Thomas, L. E.: J. Nat. Cancer Inst., **10**:1375, 1950.

to grave errors in quantitation, especially in the case of substances with a high optical density, i.e., of dark shades. An extreme example will be given to shown this point. It will be taken for granted that the sections are of a very uniform thickness, a condition not easy to fufil.

Let us assume that the field is occupied by a homogeneous colored layer the light transmission of which it 5 per cent. If the same amount of colored material is distributed in discontinuous spots occupying only half the field, transmission of the entire area will go up to 50 + 2.5 = 52.5 per cent. If

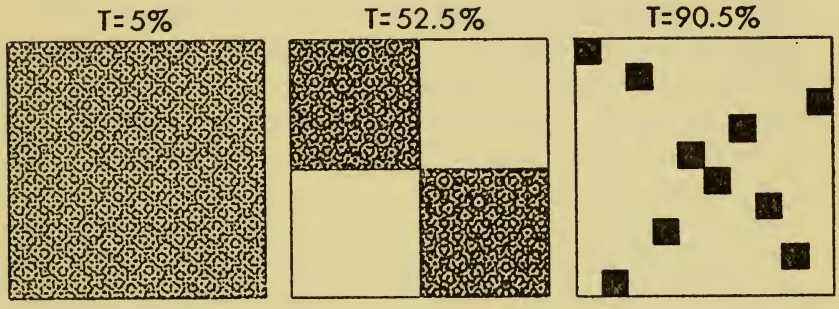


FIG. 1

it is distributed over only one-tenth of the field, transmission will rise to 90 + 0.5 = 90.5 per cent (see Fig. 1). In this way the same amount of colored matter distributed over the same area in different ways may read 5 per cent, 52.5 per cent, or 90.5 per cent, which is a spread of eighteen fold in terms of transmission.

The error decreases rapidly with increasing transparency of the color. If the transmission of the uniform layer is not 5 but 50 per cent, the corresponding readings will be 50, 75, and 95 per cent; and if the uniform transmission is 80 per cent, they will be 80, 90, and 98 per cent. However, since the absolute amount of colored matter is a function of the logarithm of transmission, the actual error in quantitation would be 30:1, 13.5:1, and 12:1, respectively.

It follows that readings of transmission obtained through a variegated area have a very limited quantitative significance, except in the case of reasonably uniform colored particles of good transparency. Such relatively favorable conditions obtain, for instance, in thin sections stained by the Bauer-Feulgen method for glycogen,[3] as could be shown by the fairly satisfactory agreement between colorimetric readings and the results of chemical analysis. The difficulties and pitfalls of quantitation by absorption colorimetry were adeptly summarized by Glick, Engström, and Malmström.[4]

The same principle holds true for semiquantitative judgments arrived at from gross inspection. A well-known example of this is the change in color of the skin of some amphibians and fish. The same fish may appear almost black or practically white, depending on the state of expansion or contraction of the chromatophores, although the amount of pigment per unit area remains unchanged.

These simple facts are often ignored in histochemistry. In a semiquantitative way, a reaction is often called intense or the tissue is stated to contain large amounts of a substance if the section shows a widespread reaction. Quantitatively, transmission of areas grossly variegated in black and white is measured by photometers, and conclusions as to the concentration of substances are drawn from the data.[5] This practice is incorrect scientifically.

For a semiquantitative (accuracy about ±50 per cent) evaluation of microscopic color reaction, model experiments can be used to great advantage. It is likely that under suitable conditions even true photometric quantitation could be achieved. This has been attempted by Marza and Chiosa[6]

3. Deane, H. W., Nesbett, F. B., and Hastings, A. B.: Proc. Soc. Exper. Biol. & Med., **63**:401, 1946.

4. Glick, D., Engström, A., and Malmström, B. G.: Science, **114**:253, 1951.

5. Cleland, K. W.: Proc. Linnean Soc., N.S. Wales, **75**:35, 1950.

6. Marza, V. D., and Chiosa, L.: Bull. d'histol. appliq. à la physiol., **12**:58, 1935.

(quantitative determination of potassium in tissue sections), but further investigations will be required before the value of this procedure is established.

For such approximate estimations, model slides serve as standards, and the Coujard technique offers a simple approach. The principles of the method will be illustrated by describing its application to the quantitation of histochemical reactions for enzymes.[7]

First of all, a highly active enzyme preparation is made according to one of the accepted methods, and its activity is assayed accurately. Serial dilutions by a factor of 2 are made with a suitable diluent, such as a 1 per cent gelatin or gum acacia solution. Standard slides are prepared as follows: carefully cleaned microscopic slides are coated thinly with egg-white glycerol, just as for histological purposes. They are subsequently heated over a Bunsen flame until completely dry. This pretreatment will prevent the running of ink when the marks are made. With a clean steel pen, marks are made on the slides, using the serial dilutions as ink. To avoid confusion, the dilution fraction can be used as a mark for each "ink." Every slide will carry the marks of the entire dilution series. The slides are dried, fixed for a few hours in alcohol or acetone, coated with thin (about 0.1 per cent) collodion, and washed. A number of them are incubated, together with the same number of regular histological slides, in the substrate solution. At intervals (e.g., 5, 10, 20, 40 minutes, etc.) one tissue slide and a corresponding standard are removed from the incubating mixture, and the color is developed. The pair of slides in which the tissue structure in question first shows up in the shade chosen (for simple inspection, preferably black; for colorimetric measurement, any shade lighter than black) is used for comparison. The mark made with the lowest dilution and showing in the shade chosen will have approximately the same activity per unit area as the histological detail in question. It is important

7. Gomori, G.: Exper. Cell Research, 1:33, 1950.

that the time at which a tissue detail *first* shows up in black should not be missed, since estimation of further increase in color development beyond the level of black is impossible. Nothing can be blacker than black.

The specific enzymatic activity (activity/ml of tissue) of a structural detail (granule, fiber, brush border, etc.) appearing in a uniform shade can be calculated on the basis of the

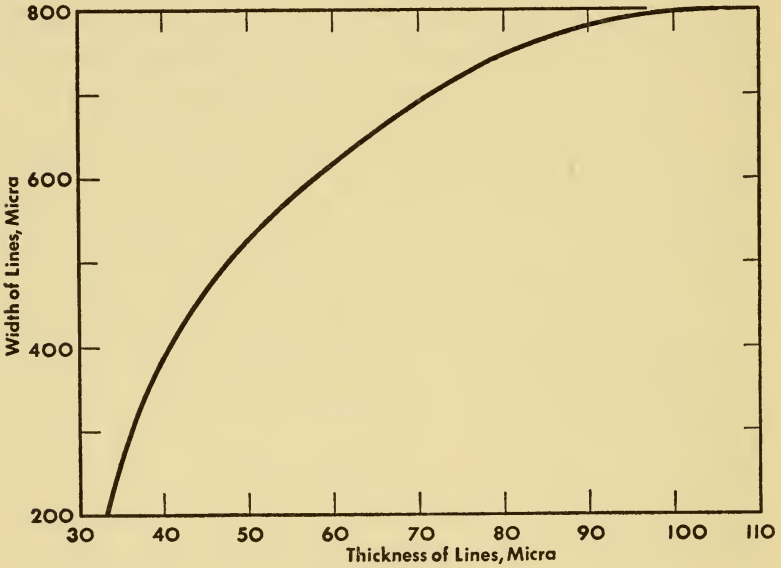


Fig. 2

principle that the colorimetric density of a homogeneous layer is proportional to the absolute amount of colored material per unit of projection area. Therefore, the specific activity of any detail will be identical with that of the mark showing up in the same shade, multiplied by H/h, where H stands for the thickness of the fluid layer of the mark and h for the thickness of the tissue section, provided that the structure occupies the entire thickness of the section. The value for H can be obtained from the empirical curve given in Figure 2, after measuring the average width of the mark

lines with an eyepiece micrometer. For the derivation of the curve and for the possible sources of error inherent in this method the reader is referred to the original paper.[7]

It should be stressed that, with methods of the type described, quantitation applies only to tissue details appearing in a uniform shade, and over-all values for the entire specimen cannot be obtained. For such over-all values some method like that of Doyle,[8] in which the reaction products are extracted from the entire section and their amount is determined chemically by test-tube assay, must be used. A new method,[9] based on the introduction of a radioactive isotope into the precipitate obtained enzymatically, also appears to be theoretically correct for over-all quantitation.

With suitable minor modifications, model experiments can be employed in several of the quantitative aspects of histochemistry.

1. The sensitivity of a method, expressed in terms of minimal concentration of a substance still giving a recognizable reaction, can be determined.

2. The concentration of chemical constituents in tissue elements can be calculated.

3. The quantitative effect of procedures of fixation and embedding, especially loss in enzymatic activity, can be studied.

4. The kinetics of enzymatic reactions in tissue sections can be followed, and results obtained with Doyle's method can be checked and supplemented.

8. Doyle, W. L.: Science, **111**:64, 1950; Doyle, W. L., Omoto, J. H., and Doyle, M. E.: Exper. Cell Research, **2**:20, 1951; and Doyle, W. L.: Quantitative aspects of the histochemistry of phosphatases, in Symposium on cytology (East Lansing: Michigan State College Press, 1951).

9. Barka, T., Szalay, S., Pósalaky, Z., and Kertész, L.: Kisérletes orvostud., p. 1, 1951.

PART II

SYSTEMATIC HISTOCHEMISTRY

The part of the book which follows contains the description and critical evaluation of histochemical techniques for various substances. No claim is made to include all techniques ever described; in fact, for each substance only one or a few methods of proved value will be recommended, although some untested methods and methods of historical or theoretical interest will also be mentioned. The techniques as given in detail do not necessarily follow the exact specifications of their authors; they may be slight modifications, found to be more satisfactory or simpler than the original procedures.

CHAPTER VI

INORGANIC SUBSTANCES

Inorganic constituents occur in tissues in three forms: (1) soluble, diffusible, and, for all practical purposes, completely ionized; (2) insoluble but readily convertible into the soluble form; and (3) incorporated into complex, soluble, but poorly diffusible or insoluble organic molecules. The last form is often called "occult" or "masked" because the regular reactions of the inorganic part are not obtainable unless the organic matrix is first more or less completely destroyed.

Diffusible substances can be localized only by the use of the freezing-drying method. Even so, localization is often only approximate on account of their high mobility, causing noticeable displacement of the solute before quantitative precipitation by the reagent can take place.

Some substances of the second group are capable of giving direct ionic reactions without being dissolved first; others must be treated with acid to make them soluble. In the latter case the reagent must be present in the acid from the very beginning, to bind the ions as fast as they are formed in the course of solution.

The demonstration of "masked" constituents presents considerable difficulties. Unmasking agents, with a few exceptions, are quite harsh chemicals (or intense heat) which may destroy tissue architecture beyond recognition.

A. METALLIC ELEMENTS

Sodium.—No method is available for the demonstration of this element. The demonstration of "sodium chloride" by the $AgNO_3$ technique[1] is such a naïve idea that it does not deserve serious consideration.

1. Seeger, P. G.: Ztschr. f. mikr.-anat. Forsch., **53**:65, 1943.

Potassium.—This element exists partly (probably to a small extent) in a poorly ionized, protein-bound, nondiffusible form, while the bulk of it is diffusible and ionized. Whether or not both fractions are demonstrable histochemically is not known. The reagent used is sodium cobaltinitrite, which forms with potassium a microcrystalline precipitate of potassium cobaltinitrite, orange in color. For better visibility it is transformed in a second step into black cobalt sulfide. The reaction is quite specific for potassium. Originally it was believed that creatine could give a spurious reaction; more recent findings, however, indicate that the precipitate was caused by traces of potassium in the creatine sample used[2] Pure creatine gives no precipitate. Localization is fair to good.

Method (Macallum's,[3] modified)

Reagent.—Solution A, dissolve 5 g. of cobalt nitrate in a mixture of 10 ml. of distilled water and 2.5 ml. of acetic acid; solution B, dissolve 15 g. of sodium nitrite in 25 ml. of distilled water. For use, pour solutions A and B together, shake for a few minutes until the bulk of the nitrous fumes which develop on mixing has escaped. Chill the reagent, one dish of distilled water and three to four dishes of 50–70 per cent alcohol in an ice bath. Place small pieces of fresh tissues in the reagent for about 2 minutes. Rinse briefly in distilled water, followed by thorough rinses in the alcohols. Transfer to a dilute solution of yellow ammonium sulfide (about 1 drop to each 5 or 10 ml. of distilled water) for about 2 minutes. Wash, counterstain as desired, dehydrate, and mount. A black, granular precipitate indicates the sites of potassium.

Thorough rinsing of the tissue in dilute alcohol is very important if one wants to avoid a disturbing gray background due to adsorbed cobalt. The mixed reagent can be kept for only a few days.

2. Macallum, A. B.: Australian J. Exper. Biol., **9**:159, 1932.
3. Macallum, A. B.: J. Physiol., **32**:95, 1905.

Another reaction for potassium has been described by Carere-Comes,[4] based on the dipicrylamine test of Poluektoff.[5]

Formalin fixation is recommended by the author. No precautions are taken in the course of further handling of the tissue. Under such conditions, only a minute fraction of potassium will remain at the original sites, and the technique would have little value even if the reaction were a satisfactory one, which it is not. If a drop of a dilute solution of KCl is mixed with a drop of the reagent on a slide, one can observe under the microscope that precipitation of the orange-red K-dipicrylamine crystals starts only after about 90 seconds, and complete precipitation requires several minutes. The crystals formed are very coarse. The criticism of Claesson[6] that this method is unsuitable for the identification and localization of potassium appears to be fully justified. The explanation of "positive reactions" is this: The reagent is a good plasma stain, antiquated now but much used during the pioneer period of histology under the name of "Aurantia." It will stain all acidophilic structures, some of which (muscle, red cells) happen to be rich in potassium.

Calcium.—Soluble calcium could possibly be demonstrated by treating frozen-dried sections with a solution of ammonium oxalate, although there is no record of this ever having been attempted. Rabl[7] proposed the use of a fixative composed of formalin and oxalate; however, localization under such conditions would be far from accurate. The octahedric crystals of calcium oxalate are easily recognizable.

For insoluble deposits of Ca salts it is important to use neutral fixatives because both Ca phosphate and carbonate are acid-soluble. Neutral formalin or alcohol or their mixtures are suitable fixatives.

4. Carere-Comes, O.: Ztschr. f. wissensch. Mikr., **55**:1, 1938.
5. Poluektoff, N. S.: Mikrochemie, **14**:265, 1933.
6. Claesson, L.: Acta anat., **3**:1, 1947.
7. Rabl, C. R. H.: Klin. Wchnschr., **2**:1644, 1923.

The methods used for the demonstration of insoluble Ca fall into two classes: (1) those which are specific for Ca itself (the gypsum, oxalate, and lake-dye reactions) and (2) those which demonstrate the anions of calcareous deposits (heavy-metal methods).

The only absolutely specific reaction is Schujeninoff's.[8] This is based on the formation of insoluble crystals of gypsum ($CaSO_4$) by the action of H_2SO_4 in a dilute alcoholic medium.

Method

Cover the section with 50 per cent alcohol; place a drop of 5–10 per cent H_2SO_4 on the under surface of a cover slip and put it on the section. Watch slide under the microscope; almost immediately typical rhombic crystals, very prone to aggregate in swallowtail or rosette-like formations, will appear.

The sections cannot be counterstained, and the crystals are far too coarse to permit exact localization.

Several hydroxyanthraquinone dyes (alizarinsulfonic acid, purpurin,[9] anthrapurpurin)[10] give intensely colored (reddish or purple) insoluble lakes with calcium and can be used for the demonstration of deposits of a medium particle size. Relatively large, dense structures such as bone spicules are poorly penetrated; in the case of fine, dustlike deposits the shades are not intense enough to be seen distinctly. This applies also to gallamin blue,[11] a lake dye of another chemical group. Anthraquinone lake dyes can be used in the form of a 0.1–0.5 per cent solution in 50 per cent alcohol; gallamin blue in 0.1–0.2 per cent aqueous solution in an M/5 borate buffer of pH 7.6. Staining time is several hours. For counterstaining, one of the plasma stains (anilin blue or light green

8. Schujeninoff, S.: Ztschr. f. Heilk. **18**:79, 1897.
9. Grandis, V., and Mainini, C.: Arch. ital. de biol., **34**:73, 1900.
10. Salomon, H.: Jahrb. f. wissensch. Bot., **54**:308, 1914.
11. Stock, A.: J. Roy. Micr. Soc., **69**:20, 1949.

for the anthraquinone lakes, eosin for gallamin blue) should be used, because basic dyes may be adsorbed to sites previously stained by the lake, giving dark, murky shades.

The method described by Crétin[12] is quite specific and gives very sharp pictures; however, the preparation of the highly unstable reagent is difficult. The method is so capricious as to be a curiosity rather than a dependable test.

Since in animal tissues almost all insoluble Ca is in the form of phosphate and carbonate and, conversely, practically all insoluble phosphate and carbonate are Ca salts, any method which demonstrates these two anions is reasonably specific for Ca. With plant tissues, which may contain large amounts of Mg phosphate, this does not hold.

The phosphates and carbonates of almost all heavier metals are insoluble, and many of them are convertible into intensely colored compounds. Thus there is a wide choice of reagents, as enumerated by Stoeltzner.[13] To mention a few: $Ag \rightarrow$ metallic Ag (black); $Co \rightarrow CoS$ (black); $Cu \rightarrow Cu_2Fe(CN)_6$ (red-brown); $Fe^{++} \rightarrow Fe_3^{++}$ $(Fe^{+++}[CN]_6)_2$ (Turnbull's blue); etc. Of these, only the silver technique is employed extensively, although at times the others may also be useful, especially when a shade other than black is desired.

The silver technique (Salge and Stoeltzner,[14] Kóssa[15]).

Method

Before using the silver solution, rinse the slide thoroughly in distilled water. Immerse the slide for 5–10 minutes in a 0.2–1 per cent solution of $AgNO_3$. At the sites of Ca phosphate-carbonate the corresponding silver salt, yellowish in color, will form. This can be reduced to metallic silver either by exposing the jar to direct sunlight (or to the light of an

12. Crétin, A.: Bull. d'histol. appliq. à la physiol., **1**:125, 1924.
13. Stoeltzner, W.: Virchows Arch. f. path. Anat., **180**:362, 1905.
14. Salge, B., and Stoeltzner, W.: Berl. klin. Wchnschr., **37**:298, 1900.
15. Kóssa, J. von: Beitr. z. path. Anat. u. z. allg. Path., **29**:163, 1901.

ultraviolet light source) until the precipitate appears black or by using photographic developers.

Reduction of the silver salt by light has certain disadvantages: (1) a brownish halo may sometimes form around the black granules, or (2) the black precipitate may turn brown when the unreacted silver is removed. For these reasons, some workers prefer to use photographic developers. The slides are first thoroughly washed in many changes of distilled water and then immersed in a dilute solution of hydroquinone or pyrogallol (about 0.5 per cent; concentration not important) for about 2 minutes and rinsed again. After both methods of reduction, the unreacted silver must be removed by a short bath in a thiosulfate (hypo) solution (about 2 per cent); otherwise the slides may darken later. After photographic development the slide presents a very sharp contrast between black and white, no intermediate shades being present; however, failure to wash the slide very thoroughly before the use of the developer will result in the deposition of a very fine, dustlike black precipitate all over the tissue. Slides prepared in either way can be counterstained as desired, dehydrated, and mounted.

The only source of error with the silver method is the presence of massive deposits of uric acid and of its salts, which may stain very much like phosphate-carbonate. For differentiation between urate and phosphate-carbonate see the section on uric acid.

The other heavy-metal techniques are performed in an analogous way. First, the section is immersed for ½–1 hour in the solution of a salt of the heavy metal (e.g., cobalt nitrate, ferrous sulfate, etc.), then washed thoroughly and treated with the reagent (ammonium sulfide, acidified potassium ferricyanide, etc.). The two main disadvantages of these techniques are that (1) the proteins may retain the heavy metal rather stubbornly and in this way give rise to a more or less intensely colored background and (2) the penetration of heavy-metal salts into dense granules or spicules

is very limited. The protein error is very marked in the case of native proteins (unfixed tissues), whereas after a good fixation it is usually negligible unless the slide is exposed to the heavy-metal salt for many hours or even days.

The intense blue staining of calcified structures, such as bone matrix, by various hematoxylin lakes (hemalum, iron, and chrome hematoxylin) is not specific for any component of bone salt.[16] Neither Ca carbonate nor phosphate will stain with hematoxylin; on the other hand, bone matrix will stain even after complete removal of bone salt by a strong acid. The affinity of bone matrix for hematoxylin lakes is due to the chemical constitution of the organic framework (presence of mucopolysaccharides?), specifically, to its ability to bind the metallic component.

Masked Ca (mainly in cell nuclei) can be demonstrated only by microincineration.

Barium and strontium.—Waterhouse[17] recommends a solution of sodium rhodizonate in distilled water or in a buffer of pH 7 as a reagent for Ba and Sr. These two ions give red-brown precipitates with rhodizonate. According to the author, the reaction with Ba can be prevented or abolished, respectively, by treating the section with a solution of sodium chromate; the reaction with Sr is not affected.

The specificity of this test requires confirmation. Waterhouse finds that, under the conditions specified, Ca will not react. This is not entirely correct; both bone salt and freshly precipitated pure Ca phosphate stain in a distinct ochre shade with rhodizonate. It is questionable whether Ba and Sr can ever be recognized by the rhodizonate test in the presence of excess Ca. Furthermore, it is known[18] that Pb

16. Schuscik, O.: Ztschr. f. wissensch. Mikr., **37**:215, 1920; Cameron, G. R.: J. Path. & Bact., **193**:929, 1930.

17. Waterhouse, D. F.: Nature, **167**:358, 1951, and Australian J. Scient. Research, B, **4**:145, 1951.

18. Feigl, F.: Chemistry of specific, selective, and sensitive reactions (New York: Academic Press, 1949).

and Hg give a precipitate of a shade very similar to that obtained with Ba.

Magnesium.—Quinalizarin, titan yellow, and a number of azo dyes have been suggested for the demonstration of this element.[19] In model experiments some of them appear to be sufficiently specific; however, in sections of animal tissues no staining can be obtained with any of them. Presumably their sensitivity is relatively low.

Iron.—This metal occurs in two forms in animal tissue. In one form, represented by hemosiderin, it behaves like any poorly soluble inorganic ferric compound, such as ferric oxide, readily demonstrable by the common reagents of analytical chemistry. In the other form (occult or masked iron), exemplified by hemoglobin, iron is a part of complex organic molecules and demonstrable only after destruction of the organic part. Iron appears to be present in the tissues exclusively in the ferric state, although there are a few reports on the finding of ferrous iron.

For the demonstration of iron, tissues should be fixed in a neutral fixative, such as neutral formalin, alcohol, or a mixture of the two. Although hemosiderin is far less acid-soluble than Ca phosphate, it is attacked to a noticeable degree by acid-containing fixatives, such as Bouin's fluid. The result is not only blurring of the picture but also a false localization. Certain morphological structures, especially nuclei[20] and, to a lesser extent, coarse connective-tissue fibers, have an amazing affinity for the ferric ion and will adsorb it even from extremely dilute solutions (Gilson,[21] Wiener[22]) and hold it tenaciously.

The oldest technique for the demonstration of iron is

19. Broda, B.: Wiadomości Farm., **63**:6 and 15, 1936; and Okamoto, K., Seno, M., and Shibata, D.: Taishitzu Gaku Zasshi, **13**:97, 1944.

20. Macallum, A. B.: Quart. J. Micr. Sc., **38**:175, 1896, and Proc. Roy. Soc. London, **50**:277, 1891.

21. Gilson, G.: Rep. British A. Adv. Sc., p. 778, 1892.

22. Wiener, A.: Biochem. Ztschr., **77**:27, 1916.

Quincke's[23] iron sulfide test (A. Mayer, 1850). It is based on the formation of green-black ferrous sulfide by the action of ammonium sulfide.

Method

Dilute ammonium sulfide (colorless or light yellow; darker samples do not work well) with 5–10 volumes of distilled water. Immerse sections for 20–30 minutes. Wash briefly under the tap; counterstain with a red nuclear stain (safranine, neutral red, lithium carmine); wash once more, dehydrate, and mount. Sites of hemosiderin iron show up in a very dense green-black shade.

This method is incompatible with the use of mercury-containing fixatives. Even after treatment with iodine, enough mercury may be left in the tissue to give a blackish precipitate with the reagent. Ferrous sulfide is quite sensitive to acids; even dilute hydrochloric acid will remove it in a matter of minutes; this property will differentiate it from the acid-resistant sulfides of other metals (lead, bismuth, etc.). Even an acidic dye solution used for counterstaining, such as alum carmine, may bleach the finest granules.

It has been asserted that the ferrous sulfide method is more sensitive than the Prussian blue method (the description of which will follow) because it unmasks certain iron compounds which do not react with the latter. However, on careful comparison of consecutive serial sections stained according to the two techniques, it becomes clear that the efficiency of the two methods is the same, except for the fact that ferrous sulfide is quite opaque, while Prussian blue is somewhat transparent.

The most reliable reagent for the hemosiderin type of iron is an acidified solution of potassium ferrocyanide (Perls).[24] Ferric ions, released by the action of the acid, are trapped by

23. Quincke, H.: Arch. f. klin. Med., **25**:567, 1880, and Arch. f. exper. Path. u. Pharmakol., **37**:183, 1896.

24. Perls, M.: Virchows Arch. f. path. Anat., **39**:42, 1867.

the ferrocyanide as fast as they are formed, provided that ferrocyanide is really present "on the premises." Since it diffuses much more slowly than the acid (and very poorly through collodion), it may arrive too late to bind the ferric ions where formed, and some diffusion of the latter may take place. For this reason, ferrocyanide must be given a head start. The following technique will invariably yield sharp pictures.

Method

Fixation in a neutral fluid. Celloidin must be removed from sections of celloidin-embedded material.

Place slides (or frozen sections) in a fresh and filtered 5–10 per cent solution of potassium ferrocyanide. After 5 minutes add about ½ volume of a 10 per cent solution of hydrochloric acid. The latter must be of a good analytical grade and contain no iron (produce no visible greenish or bluish tinge when mixed with the ferrocyanide). Stir the mixture. Keep sections in it for about 20–30 minutes. Wash under the tap. Counterstain with a red nuclear dye (alum carmine, safranine, neutral red, but *not* lithium carmine, which will bleach Prussian blue), dehydrate, and mount. Hemosiderin iron will show up in an intense blue shade. Balsam of Canada may cause gradual fading of the stain over a period of months or years; the more modern synthetic mounting media are safe.

Tirmann[25] suggested the combination of the preceding two methods, to utilize the advantages of the absolute specificity of the Prussian blue method and the purportedly higher sensitivity of the ferrous sulfide method. He proceeds as follows: The section is first treated with ammonium sulfide, washed, and then subjected to the action of an acidified solution of potassium ferricyanide, which will convert ferrous sulfide into Turnbull's blue (ferrous ferricyanide), of a shade indis-

25. Tirmann, J.: Görbersdorfer Veröffentl., **2**:101, 1898.

tinguishable from that of Prussian blue (ferric ferrocyanide). This technique is not recommended because it invariably produces minor, and sometimes major, artifacts.[26] Ferrous sulfide is attacked by the acid in an explosive way, resulting in the deformation of the solid granules into balloon- and burrlike structures with a light center and dark outlines.

It should be mentioned here that no reaction is ever obtained in animal tissues under either normal or pathological conditions if acidified ferricyanide is applied directly to the sections.[26] This shows absence of ferrous iron.

A few other methods will be mentioned briefly. Macallum[27] suggests the use of hematoxylin, which forms a blue-black lake with iron. It is easy to show that under strictly neutral conditions hematoxylin will not stain any iron in the tissues. Positive reactions are obtained only after treatment with acid (exposure to sulfuric acid–alcohol,[28] fixation in Bouin's fluid or even in unneutralized formalin). Such treatment is wrong in principle, as has been pointed out. In addition to producing diffusion artifacts, the specificity of the method is poor. Other metals such as lead and copper (under experimental and pathological conditions) will give reactions indistinguishable from that of iron. Moreover, dichromate fixation may cause unsaturated fatty substances (myelin, etc.) to stain very intensely.[29]

The thiocyanate technique[30] is unsuitable for localization because the red coloration obtained with it is highly diffusible. Schmelzer[31] recommends the application of HCNS gas to sections mounted in paraffin oil, and he claims good localization.

26. Gomori, G.: Am. J. Path., **12**:655, 1936.
27. Macallum, A. B.: J. Physiol., **22**:92, 1897.
28. Dieterle, R. R.: Arch. Path., **10**:740, 1930.
29. Mühlmann, M.: Virchows Arch. f. path Anat., **266**:697, 1927.
30. Kockel, H.: Virchows Arch. f. path. Anat., **277**:856, 1929.
31. Schmelzer, W.: Ztschr. f. wissensch. Mikr., **50**:99, 1933.

The 8-hydroxyquinoline[32] and the dinitroresorcinol[33] techniques offer no advantages.

Masked or occult iron.—A number of well-defined iron-containing compounds, such as hemoglobin, malaria pigment, formalin pigment, and possibly some other less well-known ones, do not show any reaction with the Prussian blue method; however, they can be made positive by destroying the organic part of the molecule ("unmasking" the iron). Ammonium sulfide and acids are sometimes referred to as unmasking agents, but wrongly so. They do not liberate demonstrable iron from any of the substances mentioned.

A good demasking agent must spare tissue architecture and leave the iron at its original site. No strongly acidic substance can satisfy the latter condition. The only reagents which have been used successfully are free chlorine, bromine,[34] and hydrogen peroxide.[35] Chlorine is applied either in the form of a gas (Okamoto)[36] or in a nonaqueous solvent (Kockel).[30] These methods are cumbersome and not very reliable. Even if they do work, they will transform iron into highly hygroscopic and diffusible ferric chloride, which cannot be localized with any accuracy. On the other hand, hydrogen peroxide produces insoluble ferric oxide. Whether all or only some of the iron-containing biological substances are unmasked by it and to what extent remains to be determined; hemoglobin gives a fairly intense Prussian blue reaction after treatment with hydrogen peroxide.

Method

Apply a few drops of a 30 per cent solution of hydrogen peroxide (e.g., Superoxol), alkalized with some dilute ammonia or sodium carbonate, to the section and leave it on

32. Thomas, J. A., and Lavolley, J.: Bull. d'histol. appliq. à la physiol., **12**:400, 1935.

33. Humphrey, A. A.: Arch. Path., **20**:256, 1935.

34. Klein, G.: Praktikum der Histochemie (Berlin: J. Springer, 1929).

35. Brown, W. H.: J. Exper. Med., **13**:477, 1911.

36. Okamoto, K.: Acta Scholae Med. Univ. Kioto, **20**:413, 1937.

for about 30 minutes. Wash the slide under the tap and subject it to the Prussian blue method.

Policard[37] finds that the most sensitive method to demonstrate iron is microincineration.

Copper.—According to Okamoto and co-workers,[38] certain invertebrate tissues contain some kind of cupric analogue of hemosiderin. The form in which copper occurs in the tissues of higher species is not known, but it is almost invariably masked and not demonstrable directly, except when cupric salts have been administered parenterally.

Okamoto and Utamura[39] suggest the use of two reagents for the demonstration of copper: rubeanic acid and *p*-dimethylamino-benzylidenerhodanine. The former was found in model experiments to be highly specific and sensitive, although, contrary to the findings of Okamoto,[40] positive results were never obtained in human tissues. Perhaps an unmasking pretreatment with strong hydrogen peroxide would help to reveal copper even in mammalian organs.

Method

Fixation in absolute alcohol or in formalin.

Incubate sections at 37° C. for 12–24 hours in a mixture consisting of 50 ml. of 10 per cent Na acetate and 1–3 ml. of a 0.1 per cent solution of rubeanic acid (dithiooxamide) in alcohol. Copper greenish black.

Theoretically, copper should be demonstrated also by acidified potassium ferrocyanide, in the form of reddish-brown cupriferrocyanide. However, the color would be overlaid beyond recognition by the shade of Prussian blue, since the amount of iron present in the tissues far exceeds that of copper.

37. Policard, A.: Bull. d'histol. appliq. à la physiol., **11**:216, 1934.

38. Okamoto, K., Utamura, M., and Mikami, G.: Acta Scholae Med. Univ. Kioto, **22**:335, 1938–39.

39. Okamoto, K., and Utamura, M.: Acta Scholae Med. Univ. Kioto, **20**:573, 1937–38.

40. Okamoto, K., Utamura, M., and Mikami, G.: Acta Scholae Med. Univ. Kioto, **22**:348, 1938–39.

Zinc.—Fair amounts of this element are known to occur in the pancreatic islets (as a component of insulin) and in the red cells (as a component of carbonic anhydrase).

Mendel and Bradley[41] have described a method based on the insolubility of zinc nitroprusside. It has been applied only to tissues of mollusks, which contain very large amounts of zinc.

Method

Fixation not specified in original article; presumably alcohol or formalin would do. Treat paraffin sections for 15 minutes at 50° C. with a 10 per cent solution of Na nitroprusside. Wash under the tap for 15 minutes. Mount section in water and let a drop of dilute potassium sulfide solution run under the cover slip. An intense purple shade indicates the presence of zinc.

Okamoto[42] suggests diphenylthiocarbazide as a reagent.

Method

Alcohol fixation. Prepare a saturated solution of diphenylthiocarbazide in 60 per cent alcohol. Use solution when 2–4 days old. Add 1–3 ml. of this solution to 50 ml. of a borate buffer of pH 8.4–9.2. Stain sections in the mixture for 2–3 hours at room temperature. Rinse briefly in water. Counterstain lightly with hematoxylin; do not differentiate. Mount in glycerol-jelly. Zn purplish red. Mercury gives a more bluish-purple shade.

This method has yielded good results in the rabbit pancreas;[43,44] equivocal or poor ones in the pancreases of other species.[44]

Manganese.—Grundland and Bulliard[45] suggest the use of

41. Mendel, L. B., and Bradley, H. C.: Am. J. Physiol., **14**:313, 1905.

42. Okamoto, K.: Tr. Soc. Path. Jap., **32**:99, 1942; and Okamoto, K., and Hashimoto, M.: Taishitzu Gaku Zasshi, **13**:83, 1944.

43. Kadota, J.: J. Lab. & Clin. Med., **35**:568, 1950.

44. Gomori, G.: Unpublished.

45. Grundland, I., and Bulliard, H.: Compt. rend. Soc. de biol., **142**:201, 1948.

8-hydroxyquinoline for the demonstration of manganese. In an oxidizing medium it is supposed to stain manganese brown-black. The method has not been tested on animal material, but it appears unlikely that it will prove sensitive and selective enough, especially in the presence of much larger amounts of iron.

Metals Occurring under Experimental or Pathological Conditions

THALLIUM

Method of Barbaglia[46]

Fix tissues in 95 per cent alcohol containing 2–5 per cent of iodine and 5–10 per cent of potassium iodide. Thallous iodide precipitates in the form of yellow crystals. This is an untested method.

BERYLLIUM

Method of Denz[47]

Fix in formalin or formalin-alcohol. Mix equal volumes of a fresh 0.5 per cent solution of Naphthochrome green B and of phosphate buffer pH 5.0. Stain sections in this mixture for 30 minutes at 37° C. Wash in water, differentiate in absolute alcohol. Wash once more; counterstain with acridine red. Dehydrate and mount. Be stains apple green. Iron and aluminum give somewhat different green shades. This is an untested method.

Lead.—The two methods described for the demonstration of this element are based on the insolubility of its yellow chromate and its brown-black sulfide, respectively.

The chromate method[48] is for use with tissues not requiring decalcification. The reagent (potassium dichromate)

46. Barbaglia, V.: Studi Sassari, **8**:253, 1930.

47. Denz, F. A.: Quart. J. Micr. Sc., **90**:317, 1949.

48. Crétin, A.: Compt. rend. A. anat., **16**:241, 1929; Frankenberger, Z.: Compt. rend. A. anat., **16**:241, 1921.

can be incorporated in the fixative (in the form of Regaud's mixture) or applied to sections of formalin- or alcohol-fixed material. Lead is demonstrated in the form of yellow, rather opaque crystals, soluble in dilute nitric acid and blackened by ammonium sulfide.

This method cannot be used with bones which require decalcification, since lead chromate is soluble in acids. In bones, lead can be demonstrated by transforming it into brown-black PbS (Sieber),[49] which is resistant to the action of moderately strong acids. The pieces are fixed in formalin saturated with H_2S, washed, and decalcified with 5–10 per cent trichloroacetic or formic acid. Lead will show up in a dark-brown or black shade, depending on the amount present. However, a number of heavy metals form very similar sulfides, indistinguishable from that of lead without further identifying tests.

Theoretically, it should be possible to localize lead by decalcifying the pieces of bone with 5–10 per cent sulfuric acid containing about the same concentration of sodium or ammonium sulfate. Under such conditions lead would be transformed into white, insoluble lead sulfate, which could then be identified in the form of the sulfide. Other heavy metals would not interfere.

Mercury.—The forms in which mercury may occur in the tissues are poorly known. Judging from the reactions described for its identification, it appears to be rather loosely bound and reactive.

Brandino[50] suggests the use of diphenylcarbazide as a reagent but does not give any detailed instructions. Okamoto[51] uses diphenylthiocarbazide or diphenylthiocarbazone (dithi-

49. Sieber, E.: Arch. f. exper. Path. u. Pharmakol., **181**:273, 1939.

50. Brandino, G.: Studi Sassari, **5**:85, 1927.

51. Okamoto, K., Seno, M., and Okumura, T.: Taishitzu Gaku Zasshi, **13**:89, 1944.

zone; see method under "Zinc"). Hand and associates[52] reduce mercurous mercury with 10 per cent thioglycolic acid, and both the mercurous and mercuric form with stannous chloride. The metallic mercury which appears is in the form of dark droplets. All the techniques mentioned require thorough testing for specificity.

Bismuth, silver, and gold.—After being introduced into the animal organism, these metals are converted in a short time into dark-brownish or blackish, granular, rather unreactive compounds the chemical nature of which is not well understood (proteinate, sulfide, or reduced metal?).

The identification of heavy metals in the tissues is a problem of analytical chemistry rather than of histochemistry. In histochemical studies the identity of the metal to be demonstrated is usually known in advance (having been introduced artificially). Therefore, the task is not so much to differentiate between two or more metals as to establish the metallic nature of the pigment granules and to distinguish them from other colored granular substances such as soot, melanin, formalin pigment, pigment of wear and tear, etc. In most cases purely morphological criteria (type of cells in which the granules are seen) will do more than histochemical tests to decide this question.

The following relatively simple tests are recommended:

1. Apply 30 per cent hydrogen peroxide to the section for 5 minutes. Silver and gold remain unchanged (the latter will actually darken; method of Elftman);[53] bismuth[54] and most other pigments will be bleached.

For the identification of bismuth, rinse the bleached slide thoroughly and proceed according to one of the following two methods:

52. Hand, W. C., Edwards, B. B., and Caley, E. R.: J. Lab. & Clin. Med., **28**:1835, 1943.

53. Elftman, H., and Elftman, A. G.: Stain Technol., **20**:59, 1945; Elftman, H., Elftman, A. G., and Zwemer, R. L.: Anat. Rec., **96**:341, 1946.

54. Wachstein, M., and Zak, F. G.: Am. J. Path., **22**:603, 1946.

a) Method of Komaya[55]

Make up two solutions: solution A, 0.5 g. of quinine sulfate or hydrochloride in 25 ml. of 2–3 per cent H_2SO_4; solution B, a 5 per cent solution of potassium iodide. For use, mix equal parts of solutions A and B and pour the mixture over the slide. After 5–10 minutes, decant the reagent, counterstain with a dilute (about 0.05 per cent) solution of light green to which a few drops of the reagent are added. Blot section, dehydrate, and mount it in balsam. Bismuth orange-red.

b) Method of Castel[56]

Same as that of Komaya, except that quinine is replaced by brucine. Results practically the same with both methods.

2. Treat section with a dilute solution of bromine (dissolve a few crystals each of KBr and of $KBrO_3$ in a Coplin jarful of distilled water and add a few drops of concentrated HCl). Granules of gold and bismuth are completely dissolved in 10–15 minutes; silver is bleached (formation of bromide). Wash the slide; pour over it any photographic developer; silver bromide will be reduced to black metallic silver.

B. NONMETALLIC ELEMENTS

Chlorine.—The chloride ion can be localized in frozen-dried material only.[57] The reagent is a dilute solution of $AgNO_3$ (0.1–2 per cent; concentration not important), preferably in 70–95 per cent alcohol. Cl^-, $CO_3^=$, and $HPO_4^=$ will all be precipitated by it; however, dilute (about 0.5 per cent) nitric acid will remove phosphate and carbonate, and only chloride will remain undissolved. This can then be reduced to metallic silver, either by exposure to direct sunlight or by photographic developers (see under "Calcium").

55. Komaya, G.: Arch. f. Dermat. u. Syph., **149**:277, 1925.

56. Castel, P.: Bull. d'histol. appliq. à la physiol., **13**:290, 1936.

57. Gersh, I.: Proc. Soc. Exper. Biol. & Med., **38**:70, 1938.

Active chlorine (Cl_2) can be demonstrated with the method of Ferguson and Silver.[58]

Method

Fix small tissue fragments by dipping them in boiling formalin for a few seconds. Cut frozen sections 25 μ thick. Immerse sections in the following reagent for 15–25 seconds: dissolve 0.1 g. of *o*-tolidine and 1 g. of citric acid in 20 ml. of water; fill up to 100 ml. Mount in glycerol. Greenish color indicates small amounts; yellow and orange, larger amounts of free Cl_2.

Iodine.—There are no good methods available either for the iodide ion (see Gersh and Stieglitz)[59] or for occult iodine (e.g., in thyroxine).

Phosphorus.—The demonstration of the phosphate ion was described in the section on calcium. It should be added here that, whereas the usual heavy-metal techniques do not differentiate between phosphate and carbonate, there is a theoretical possibility of distinguishing between the two by the use of uranyl salts. These will give a precipitate with phosphates but not with carbonates (uranyl carbonate being fairly soluble). Insoluble uranyl phosphate is convertible into reddish-brown uranyl ferrocyanide.

Lilienfeld and Monti[60] have proposed the demonstration of organic phosphates by hydrolyzing them with nitric acid in the presence of ammonium molybdate and then reducing the phosphomolybdate precipitate in a second step to molybdenum blue. This method is worthless histochemically. Even if inorganic phosphate were the first soluble phosphorus-containing compound liberated (and this is not in the least likely), the method could not possibly localize it. First of all, ammonium phosphomolybdate is not insoluble enough;

58. Ferguson, R. L., and Silver, S. D.: Am. J. Clin. Path., **17**:35, 1947.
59. Gersh, I., and Stieglitz, E. J.: Anat. Rec., **56**:185, 1933.
60. Lilienfeld, L., and Monti, A.: Ztschr. f. wissensch. Mikr., **9**:332, 1892.

second, it does not precipitate fast enough; and, third, molybdenum blue is very soluble and diffusible and has a great affinity for protein substances.[61] Therefore, the best one could expect of this method would be a diffuse blue coloration around areas of very high concentration of organic phosphate without any sharpness of localization. The same criticism applies to all other methods based on the molybdenum blue principle (Angeli,[62] Serra,[63] and Okamoto[64]).

On the other hand, it might well be possible to develop a histochemical adaptation of Mandel and Neuberg's idea[65] (destroying organic phosphates with strong H_2O_2).

Sulfur.—Macallum[66] demonstrates sulfates by treating frozen sections of fresh tissue with a dilute solution of lead acetate, washing them with dilute nitric acid to remove all lead precipitates except acid-insoluble sulfate, and converting the latter with ammonium sulfide to brown-black PbS. Because of the great mobility of sulfate ions, this method should be applied to frozen-dried tissues.

According to Klein,[67] a number of organic compounds of sulfur are converted to sulfate by strong H_2O_2. There is a possibility that a histochemical method could be developed on this basis.

Arsenic.—The methods proposed for the demonstration of inorganic arsenic are based either on the formation of As_2S_3[68]

61. Bensley, R. R.: Biol. Bull., **10**:49, 1908.

62. Angeli, B.: Riv. di biol., **10**:702, 1928.

63. Serra, J. A., and Queiroz Lopes, A.: Portugal. Acta biol., **1**:111, 1945.

64. Okamoto, K., Seno, M., and Kato, A.: Taishitzu Gaku Zasshi, **13**:97, 1944.

65. Mandel, J. A., and Neuberg, C.: Biochem. Ztschr., **71**:196, 1915.

66. Macallum, A. B.: Die Methoden der biologischen Mikrochemie, in Abderhalden's Handb. d. biol. Arbeitsmeth., **V-2**:1099 (Berlin and Vienna: Urban & Schwarzenberg, 1912).

67. Klein, G.: Oesterreich. bot. Ztschr., **76**:15, 1927.

68. Brünauer, S. R.: Arch. f. Dermat. u. Syph., **129**:186, 1921; Osborne, E. D.: Arch. Dermat., **12**:773, 1925; Memmesheimer, A. M.: Dermat. Ztschr., **54**:4, 1928.

by H_2S or on the precipitation of arsenites and arsenates by copper sulfate in the form of the corresponding insoluble cupric salts.[69] These methods lack all specificity. Tannenholz and Muir[70] have shown that the yellow precipitate obtained after treatment of tissues with H_2S is neither As_2S_3 nor any other compound of arsenic; as far as the copper method is concerned, it would give positive reactions also with fatty acids, phosphates, carbonates, and many proteins.

69. Castel, P.: Bull. d'histol. appliq. à la physiol., **13**:106, 1936.
70. Tannenholz, H., and Muir, K. B.: Arch. Path., **15**:789, 1933.

CHAPTER VII

ORGANIC SUBSTANCES

A. SACCHARIDES

SUGARS in their free form cannot be localized histochemically, not even in frozen-dried material, because all known reactions for them are far too slow. Very recently, Okamoto, Kadota, and Aoyama[1] have suggested a new method based on the insolubility of Ba-glucose and Ba-lactose in alcohol.

Method

Fix very thin slices of fresh tissue in methyl alcohol saturated with barium hydroxide for about 24 hours at icebox temperature. Dehydrate with two or three changes of absolute alcohol, carry tissue through two changes of chloroform, and embed in paraffin. Mount paraffin sections directly on the slide; use a minimum of egg-white–glycerol. Deparaffinize section with chloroform, rinse in absolute alcohol, and transfer to a 1 per cent solution of $AgNO_3$ in 80–90 per cent alcohol; expose jar to direct sunlight. The insoluble precipitate of Ba-sugar will be transformed into the corresponding Ag compound and the latter reduced to metallic silver. Rinse slide in distilled water, remove unreduced silver with a dilute solution of Na thiosulfate; counterstain and mount. A slide rinsed thoroughly in distilled water before the silver bath can serve as a control; only the difference between the two slides is due to the presence of glucose or lactose.

This is an untested method. It sounds promising, although the accuracy of localization is probably only approximate. Phosphates are very likely to give false positive reactions. The method should be tried also on frozen-dried material.

1. Okamoto, K., Kadota, I., and Aoyama, Z.: Taishitzu Gaku Zasshi, **14**:35, 1948.

Carbohydrates occur in four types of substances in a form insoluble enough to be demonstrated even by relatively slow reactions. The chemical nature and physiological role of these substances vary widely; the only reason for their being dealt with in one section is the fact that they all contain sugar and that they can be demonstrated by the reactions of the sugar moiety. For the sake of brevity, they will be called "saccharides." Some of these substances have additional identifying reactions, due to other chemical or physicochemical features of their molecules.

Classification of the Saccharides

1. Simple polysaccharides are built up from sugar molecules only. In animals the building stones are glucose (glycogen) or galactose (galactogen); in plants they may be glucose (cellulose) or pentoses. Substances belonging in this group are either insoluble or soluble in a colloidal form only. Polymer linkages holding the individual molecules together go from C_1 to C_4 of the next molecule, although in some cases the linkages may connect to C_3. This last type of compounds shows a histochemical behavior different from that of the other members of the group.

2. Mucoid substances are characterized by their content of aminosugar (glucosamine, acetylglucosamine, or acetylgalactosamine) as the most typical component. K. Meyer[2] divides this group into three classes.

A. Mucopolysaccharides (protein-free)
   a) Neutral, containing no acid groups (example: chitin, composed of acetylglucosamine only)
   b) Acid
      1. Simple; acid component, uronic acid (example: hyaluronic acid, composed of acetylglucosamine and glucuronic acid)
      2. Complex; acid component, uronic acid and sulfuric acid or phosphoric acid (examples: corneal mucoid, hog gastric mucin, heparin, all composed of glucosamine or acetylglucosamine, some uronic acid, and sulfuric acid; chondroitinsulfuric acid, composed of acetylgalactosamine, glucuronic

2. Meyer, K.: Adv. Protein Chem., **2**:249, 1945.

acid, and sulfuric acid; certain bacterial acid polysaccharides containing phosphoric acid)

B. Mucoproteids, containing more than 4 per cent aminosugar
   a) Soluble neutral (gonadotrophins, etc.)
   b) Insoluble neutral (ovomucin)
   c) Acid (submaxillary mucoid)

C. Glycoproteins, containing less than 4 per cent aminosugar (ovalbumin, serum albumin)

3. Glycolipids[3] (cerebrosides) are complex substances which on hydrolysis yield a nitrogenous base (sphingosine), a long-chained fatty acid, and a sugar (usually galactose but in some cases glucose). The sugar is linked to the sphingosine molecule glycosidically, and the fatty acid is attached to the amino group of sphingosine. These substances are insoluble in water, ether, and petroleum ether but are soluble in pyridine and hot alcohol.

Another carbohydrate-containing lipid substance is lipositol, which contains inositol, galactose, fatty acids, phosphoric acid, and ethanolamine.

4. Nucleic acids are high polymer molecules, the individual building blocks of which are the nucleotides. The nucleotides are phosphoric esters of nucleosides, the latter being N-glucosides of purine and pyrimidine bases.

It has been known for over fifty years that there are two different kinds of nucleic acids. One of them, thymonucleic acid, is the typical component of cell nuclei; its sugar moiety is desoxyribose (hence desoxyribose nucleic acid, DNA). The other one, formerly referred to as yeast nucleic acid, occurs in cytoplasmic structures; its sugar component is ribose (hence ribose nucleic acid, RNA). Both nucleic acids are bound to proteins in an insoluble form.

General Principles of the Histochemical Demonstration of Saccharides

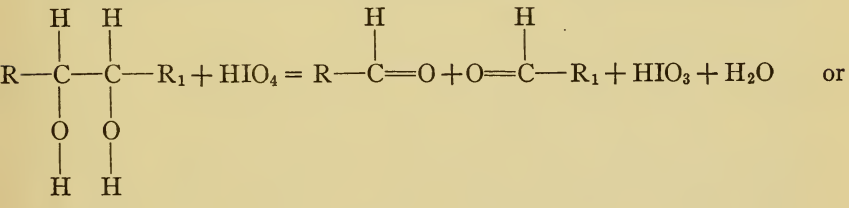
Aldehyde reactions are used to demonstrate sugars histochemically. Besides carbohydrate substances, the only other

3. Everett, M. R.: Medical biochemistry (2d ed.; New York and London: Paul B. Hoeber, 1946).

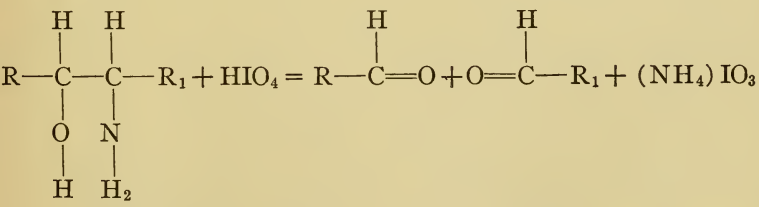
known potential aldehydes occurring in the animal body are certain lipid substances and the unidentified aldehyde of elastic tissue. Whenever an aldehyde reaction is obtained under specific conditions in a tissue defatted with lipid solvents, it is attributed to the presence of carbohydrate.

Aldehyde reactions are not given directly by any of the carbohydrate substances occurring in the tissues. The aldehyde groups have to be "liberated" first by certain chemical agents, oxidative or hydrolytic. In the case of oxidants, "liberation" is a deceptive term to describe their action. True, all aldoses have a potential aldehyde group, masked by the pyranose or furanose ring formation. However, it is not this aldehyde group which is "set free" or "revealed" oxidatively but rather entirely new aldehyde groups which are created in the middle of the carbon chain of the sugar molecule.

The action of one of the oxidants, periodic acid, is fairly well understood since the studies of Malaprade,[4] Jackson and Hudson,[5] Nicolet and Shinn,[6] Hotchkiss,[7] and others. It consists of the breaking of the carbon chain through a glycolic or HO–C–C–NH_2 group and in the oxidizing of the broken ends to aldehyde groups according to the following schemes:

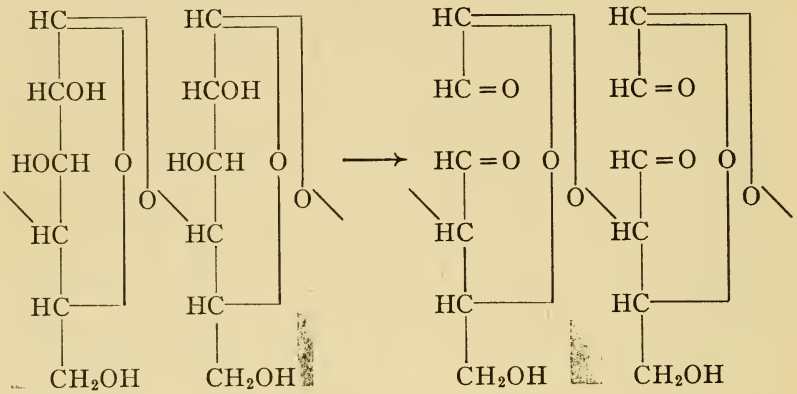


or



4. Malaprade, L.: Bull. soc. chim. France, **43**:683, 1928; Malaprade, L.: *ibid.*, ser. 5, **1**:833, 1934.
5. Jackson, E. L., and Hudson, C. S.: J. Am. Chem. Soc., **60**:989, 1938.
6. Nicolet, B. H., and Shinn, L. A.: J. Am. Chem. Soc., **61**:615, 1939.
7. Hotchkiss, R. D.: Arch. Biochem., **16**:131, 1948.

In the case of polysaccharides, treatment with periodic acid will not cause depolymerization of the molecule because the carbohydrate units remain connected by C_1–C_4 linkages, as indicated in the following scheme:



The hydroxyl and/or amino groups must be free to be attacked by periodate; if they are tied down by substitution or by any kind of linkage, the compound will not be touched. The scheme is very general and applies to all sorts of compounds possessing vicinal –OH or –OH and $–NH_2$ groups, whether or not they are of carbohydrate nature. Some of the noncarbohydrate substances oxidized to aldehydes are the amino acids serine, threonine, and hydroxylysine. The first two would not be attacked while incorporated within peptide chains unless they occupied a terminal position and were thus engaged through their carboxyl groups only. Some carbohydrates are not attacked because they lack the vicinal –OH groups (desoxyribofuranose); in other cases the individual sugar molecules do have such groups, but they are tied down in polymeric linkages (ribose nucleic acids; C_3-linked polymers such as agar[8] or heparin).[9] But even sugars which seem to possess all the necessary prerequisites for

8. Jones, W. G. M., and Peat, S.: J. Chem. Soc., p. 225, 1942.

9. Meyer, K.: Ann. New York Acad. Sc., **52**:943, 1950; Meyer, K., and Odier, M.: Experientia, **2**:311, 1946.

being oxidized by periodate may prove exceptionally resistant[10] (cellobiose, some methylglycosides); the reasons for this irregular behavior are not clear.

The amount of aldehyde formed from C_4-linked polysaccharides does not depend on the degree of polymerism, because of the availability of two vicinal OH groups (C_2–C_3) in every one of the carbohydrate units. On the other hand, in C_3-linked polysaccharides, only the terminal units can be oxidized. For this reason, the relative yield of aldehyde will increase with decreasing chain length.

The mechanism of other oxidants has not been studied in sufficient detail, but it seems to be essentially similar to that of periodate, although there are minor quantitative or qualitative differences. Periodate appears to be the most specific for glycolic linkages proper; the aldehyde groups are not further oxidized, even on prolonged exposure to the oxidant. Chromic acid, another popular oxidant, is less energetic as far as attacking *all* glycolic linkages is concerned; its action is limited almost exclusively to glycogen and mucin; glycoproteins are quite resistant to its action. The aldehyde groups are destroyed after prolonged exposure and cannot be demonstrated after a certain time. Acidified permanganate, as used by Casella,[11] combines the properties of periodate and chromic acid in that it seems to attack all glycol linkages but, on prolonged action, destroys the aldehydes formed.

In the case of nucleic acids, aldehydes cannot be produced by oxidative procedures. As mentioned before, desoxyribofuranose, the carbohydrate component of DNA, does not possess a glycolic group. In ribofuranose, the carbohydrate moiety of RNA, phosphoric acid residues in position 3 block the reaction. If the latter are removed by mild hydrolysis, the depolymerized nucleic acid becomes diffusible and will be lost from the section.

10. Jeanloz, R.: Science, **111**:289, 1950.
11. Casella, C.: Anat. Anz., **93**:289, 1942.

Feulgen and Rossenbeck[12] in 1924 discovered that thymonucleic acid yields on hydrolysis with dilute mineral acids a substance which gives an intense aldehyde reaction, reproducible both in the test tube and in microscopic sections ("nucleal reaction"). The mechanism of this reaction has been considered a mystery until very recently. According to the latest reports of Overend and Stacey,[13] a large percentage of desoxyribose and a few other carbohydrates exist naturally in a noncyclic form which gives direct aldehyde tests (due to a terminal –CH=O group). This feature distinguishes them sharply from the more common hexoses and pentoses which have no overt aldehydic character. By controlled acid hydrolysis it is possible to break just a sufficient number of glycosidic linkages to expose enough aldehyde groups for visualization of the reaction and at the same time retain much of the polymerism and insolubility of the acid on which good histochemical localization depends. Ribonucleic acid gives no reaction under such conditions.

The free aldehyde groups can be demonstrated by a number of specific aldehyde reagents. Theoretically, any aldehyde reaction used in organic analysis could be employed; in practice, however, only a few have the desirable features of a histochemical test.

The most important reagent for aldehydes is Schiff's reagent, fuchsinsulfurous acid.

The mechanism of the reaction has been clarified by Wieland and Scheuing.[14] In an acid solution, fuchsin is transformed by an excess of SO_2 into a colorless N-sulfinic acid which forms highly colored addition complexes with aldehydes. The addition complexes, of a purple shade different from that of fuchsin itself, have an excellent staining power and, being usually more insoluble than the corresponding

12. Feulgen, R., and Rossenbeck, H.: Ztschr. f. physiol. Chem., **135**:203, 1924.

13. Overend, W. G., and Stacey, M.: Nature, **163**:538, 1949.

14. Wieland, H., and Scheuing, G.: Ber. deutsch. chem. Gesellsch., B, **54**:2527, 1921.

aldehyde, cling very tenaciously to the structures stained. Schiff's reagent can be recolorized also by a different mechanism and with different end-products. The reagent is stable only in the presence of an excess of SO_2 and at a high acidity. Anything that removes or oxidizes SO_2 or reduces acidity will break the linkage between fuchsin and sulfurous acid and restore the original dye. This type of recolorization, not likely to take place under the conditions of a correctly performed histochemical test, is called a "pseudo-reaction."[15] It should not be confused with the genuine one, which is practically specific for aldehydes. Lison lists a few nonaldehydic substances which may give colorations very similar to those occurring with aldehydes; however, they are almost all nonphysiological substances, not found in animal tissues. The red shade of the pseudo-reaction is readily extracted by acids and alcohol (except in the case of acid-fast structures), while the aldehyde-regenerated dye is extremely resistant to both agents.

It must be mentioned here that not only Schiff's reagent but also undecolorized fuchsin is able to combine with aldehydes and yield purplish dyes which have staining properties different from those of fuchsin itself. Such dyes are used in Fite's modification[16] of the Ziehl-Neelsen stain, for the staining of nuclei (DeLamater)[17] and in the aldehyde-fuchsin technique[18] for elastic fibers. In periodate-treated tissues a fairly good localized staining of the aldehydes may be obtained by fuchsin proper (Arzac);[19] however, the use of Schiff's reagent is always preferable.

Another good analytical reagent for aldehydes is an alkaline solution of silver nitrate, first utilized for histochemical

15. Lison, L.: Bull. d'histol. appliq. à la physiol., **9**:177, 1932.
16. Fite, G. L.: J. Lab. & Clin. Med., **25**:743, 1940.
17. DeLamater, E. D.: Stain Technol., **23**:161, 1948.
18. Gomori, G.: Am. J. Clin. Path., **20**:665, 1950.
19. Arzac, J. P.: Analecta Med., **9**:15, 1948; Arzac, J. P.: Stain Technol., **25**:187, 1950; Arzac, J. P.: J. Nat. Cancer Inst., **10**:1341, 1950.

purposes by Bignardi[20] and Clara[21] and later described independently also by Gomori,[22] who was not then familiar with Bignardi's work. Two more silver techniques for glycogen and mucin were published by Mitchell and Wislocki[23] and by Pritchard;[24] however, these latter are silver-impregnation methods rather than histochemical tests.

At this point the difference between the argentaffin reaction and simple impregnation by silver (argyrophilia) must be pointed out. Lison[25] has emphasized that some substances (ascorbic acid, polyphenols, aldehydes, uric acid, etc.) have the ability to reduce silver solutions under specific conditions. This is the "argentaffin reaction" which may be used for the histochemical characterization of reducing substances. It should be distinguished sharply from impregnation by silver such as is obtained in various techniques for nerve and reticulum fibers, etc. In such techniques the silver is not reduced by substances contained in the tissue itself but by extraneous reducers (formalin, hydroquinone, etc.). Some of these methods are remarkably selective for various morphological structures but cannot be called "specific" in the chemical sense of the word.

The silver technique for aldehydes can be used for glycogen and mucin-like substances only, not for DNA. The reason for this is not clear; it seems that acid hydrolysis makes DNA somewhat alkali-soluble, and it will diffuse out of the section before reduction of the silver can take place.

Preparation of the Reagents

1. *Periodic acid.*—A 0.25–0.5 per cent solution in distilled water will serve all purposes. The use of alcoholic or buffered

20. Bignardi, C.: Atti Soc. nat. e mat. Modena, **70**:97, 1939; Bignardi, C.: Boll. Soc. med.-chir. Pavia, **54**:799, 1940; Bignardi, C.: Atti Soc. ital. sc. nat., **79**:23, 1940.

21. Clara, M.: Ztschr. f. mikr.-anat. Forsch., **47**:183, 1940.

22. Gomori, G.: Am. J. Clin. Path., **10**:177, 1946.

23. Mitchell, A. J., and Wislocki, G. B.: Anat. Rec., **90**:261, 1944.

24. Pritchard, J. J.: J. Anat., **83**:30, 1949.

25. Lison, L.: Histochimie animale (Paris: Gauthier-Villars, 1936).

aqueous solutions offers on obvious advantages. If loss of water-soluble matter is feared, the slide should be protected with collodion.

2. *Chromic acid.*—A 4–5 per cent solution of chromium trioxide in distilled water is used.

3. *N HCl.*—Dilute 10 ml. of concentrated HCl (assay, 36–39 per cent) with 100 ml. of distilled water.

4. *Schiff's reagent.*—There are a number of formulas[26–29] published which all give essentially identical results. Slight variations in the results are due to differences between various batches of dye and to the age of the solution rather than to minor departures from any of the formulas. The following procedure by Coleman,[28] recommended by Lillie, was found to be simple and reliable:

Dissolve 1 g. of basic fuchsin (C.I. No. 677) in 200 ml. of boiling distilled water. Cool to about 50° C. Add 1 ml. of concentrated hydrochloric acid (assay, 36–39 per cent) and 2 g. of either sodium bisulfite ($NaHSO_3$) or potassium metabisulfite ($K_2S_2O_5$); shake the flask, stopper it tightly, and allow it to stand at room temperature for 24 hours. The dye will be decolorized to a straw yellow or light-brownish solution with or without some precipitate floating in it, depending on the quality of the dye used. Add about 0.5 g. of adsorbent charcoal (e.g., Norit), shake, and filter the mixture. The filtrate must be crystal clear and colorless; should it have a light pinkish tinge, add hydrochloric acid drop by drop until all trace of color is discharged. Keep reagent in the icebox.

Some batches of reagent will remain perfectly usable for over a year; others, for reasons completely unknown, may deteriorate in a matter of weeks. The source of the dye does not seem to make much difference; reagents of highly variable keeping properties may be prepared from the same commer-

26. Wermel, E.: Ztschr. f. Zellforsch. u. mikr. Anat., **5**:400, 1927.

27. De Tomasi, J. A.: Stain Technol., **11**:137, 1936.

28. Coleman, L. C.: Stain Technol., **13**:123, 1938.

29. Rafalko, J. S.: Stain Technol., **21**:91, 1946; Lillie, R. D.: Stain Technol., **26**:163, 1951.

cial brand of fuchsin. There is nothing in the gross appearance of the reagent to indicate spoilage; this manifests itself only by unsatisfactory staining results. Entirely colorless, clear solutions may have lost all their staining power; grossly turbid or pinkish solutions, after filtration or the addition of some more bisulfite for decolorization, may work well. It is always advisable to keep a few slides, known to contain glycogen or mucin, handy and perform a test run before the main experiment.

Wermel's solution, although more complicated to prepare than the simple Schiff's reagent, is distinctly more stable than the latter.

5. *The silver solution.*—Either of the following two solutions can be used:

A) *Fontana's*[30] *silver solution.*—To a 2–3 per cent solution of silver nitrate add, drop by drop and under continuous shaking, strong ammonia water until the initial brown-gray precipitate dissolves. To the clear mixture add some more silver solution, drop by drop and under continuous shaking. The turbidity resulting from the first few drops will disappear easily; continue to add silver solution until a minimal opalescence persists. The solution will keep in the refrigerator for a few days.

B) *Methenamine-silver stock solution.*[22]—Add 5 ml. of a 5 per cent $AgNO_3$ solution to 100 ml. of a 3 per cent methenamine solution, shake until the initial heavy white precipitate disappears. This mixture will keep in the refrigerator for many months.

Fontana's solution has a tendency to produce a fine dust-like precipitate all over the slide and a fairly intense background staining. Methenamine-silver is practically free from these drawbacks; the pictures obtained with its use are very clear.

30. Fontana, A.: Dermat. Ztschr., 46:291, 1925–26.

Methods for saccharides

I. SUBSTANCES OTHER THAN NUCLEIC ACIDS (GLYCOGEN, MUCIN, GLYCOPROTEIDS, ETC.)

Fixation and embedding.—While for mucinoid substances almost any fixation will do, the correct fixative for glycogen has been the subject of numerous publications,[25, 31–32] and there is still no consensus. Most textbooks on microtechnique recommend alcohol or alcoholic mixtures on the principle that glycogen is insoluble in them while it is soluble in most aqueous fixatives. Theoretically, as applied to pure glycogen, this is a perfectly valid consideration; however, in tissues where glycogen is embedded in a complex mixture of proteins and lipids the situation is different. Any good protein precipitant will coat the glycogen particles with a protein membrane which is impermeable to the large molecules of glycogen, thus keeping them *in situ*. Lison[25] quotes the studies of Pasteels and Leonard, who state that one of the best fixatives is Bouin's fluid. Lillie,[32] on the other hand, finds that Bouin's fluid is an unsuitable fixative for glycogen. The writer agrees with the French workers on the excellence of Bouin's fluid and with Lillie on the poor results obtained with fixatives containing mercury salts. In summary, it may be said that any good histological fixative, with the exception of sublimate-containing mixtures, can be used. The best are those which act fast and produce considerable hardening of the tissues (Bouin's fluid, formalin-alcohol with or without acetic acid, etc.). However, the morphology of glycogen will vary somewhat with different methods of fixation. In general, alcoholic or acid-containing fixatives cause the aggregation of glycogen into fairly coarse droplets, while simple formalin or formalin-bichromate mixtures reveal a more uniform, fine-

31. Bensley, C. M.: Stain Technol., **14**:47, 1939; Deane, H. W., Nesbett, F. B., and Hastings, A. B.: Proc. Soc. Exper. Biol. & Med., **63**:401, 1946.

32. Lillie, R. D.: Bull. Internat. A. M. Mus., **27**:23, 1947; Vallance-Owen, J.: J. Path. & Bact., **60**:325, 1948.

ly granular distribution, resembling that seen in frozen-dried preparations.[32] The temperature of fixation seems to be of considerable importance. Liver contains a powerful glycogenolytic enzyme system, which, unless the tissue is chilled promptly, may cause a considerable loss of glycogen, especially in the interior of thick blocks. This loss may be quite conspicuous in sections stained with Bauer's[33] or Best's[34] methods, while it may not be noticeable at all if the Hotchkiss-McManus[7, 35] technique is used—an indication of a change in the constitution of the molecule rather than of actual disappearance. Although the glycogen found in other tissues is much more stable, it is safer to perform fixation of all tissues in the refrigerator.

Frozen sections, as a rule, cannot be used for the staining of glycogen. Celloidin- or paraffin-embedding are equally good.

A) *Methods specific for the group in general.*—These methods are based on aldehyde reactions after oxidative pretreatment.

Choice of the oxidizing agent.—The two oxidizers most often employed are chromic acid and periodic acid. Chromic acid is the agent of choice if a selective staining of glycogen and/or mucin is desired. Besides these two substances, starch, galactogen, cellulose, tunicin, chitin, and colloid of the thyroid follicles will also react more or less strongly after chromic acid oxidation. Glycoproteins of the connective tissue remain almost completely unstained, and for this reason the contrast between glycogen and/or mucin and the background is quite sharp. It is a curious fact that Bauer[33] in his original paper on the chromic acid–Schiff method explicitly states that mucin does not stain; all subsequent workers have found that it stains quite intensely. After periodate oxidation, all the substances previously mentioned will react, and, in

33. Bauer, H.: Ztschr. f. mikr.-anat. Forsch., **33**:143, 1933.
34. Best, F.: Ztschr. f. wissensch. Mikr., **23**:319, 1906.
35. McManus, J. F. A.: Nature, **158**:202, 1946.

addition, a number of others, such a fibrin, hyaluronic acid, various undefined glycoproteids of connective tissue, polysaccharides of bacteria, and fungi,[36] kerasin in Gaucher's disease,[37] lipofuscin,[38] ceroid,[39] and an unidentified substance in the foamy cells of Whipple's disease.[40] In general, the purple-red shade obtained after periodate oxidation is much more brilliant than that obtained after chromic acid. The permanganate method of Casella[11] is not standardized well enough to be recommended for routine use.

Some mucoid substances which stain faintly, irregularly, or not at all by the aldehyde techniques are heparin (mast cell granules; marked species differences), chondroitin-sulfuric acid (cartilage ground substance), and amyloid.

Choice of the aldehyde reagent.—Schiff's reagent gives equally good results after any of the oxidants; silver solutions are less specific and less reliable after periodate and should be used only in combination with chromic acid. The deep black shade may be an advantage in microphotography.

Methods

Oxidation with chromic acid.—Treat sections for 40–60 minutes with a 4–5 per cent solution of chromic acid. Wash under the tap for at least 5 minutes; the color of chromic acid must be removed completely.

Oxidation with periodic acid.—Treat sections for about 10 minutes with a 0.25–0.5 per cent solution of periodic acid; wash under the tap for 10 minutes. Hotchkiss[7] originally recommended a rinse in bisulfite after oxidation. The effect of this step will be a delayed and gradual development of color in the next step. This may be a desirable feature if the aim is a sharper differentiation between the more promptly re-

36. Kligman, A. M., and Mescon, H.: J. Bact., **60**:415, 1950; Kligman, A. M., Mescon, H., and DeLamater, E. D.: Am. J. Clin. Path., **21**:86, 1951.
37. Morrison, R. W., and Hack, M. H.: Am. J. Path., **25**:597, 1949.
38. Lillie, R. D.: Anat. Rec., **108**:239, 1950.
39. Lee, C. S.: J. Nat. Cancer Inst., **11**:339, 1950.
40. Black-Schaffer, B.: Proc. Soc. Exper. Biol. & Med., **72**:225, 1949.

acting substances (glycogen and mucin) and carbohydrates of the connective tissue.

Staining with Schiff's reagent.—Stain sections for about 10–15 minutes in Schiff's reagent (straight or diluted with an equal volume of water). Usually very little staining is seen during this step, especially after chromic acid oxidation. Rinse the reagent off; flood sections for 1 or 2 minutes with a 1–3 per cent solution of Na bisulfite. Wash under the tap for about 5–10 minutes. It is during this washing that the staining of mucin, glycogen, etc., in an intense purplish-red shade becomes more apparent. Counterstain with hematoxylin (staining after chromic acid often rather poor). Dehydrate and mount.

Staining with methenamine-silver.—Rinse slides thoroughly in distilled water. Incubate them at a temperature of 37°–50°C. in a mixture consisting of 25 ml. each of methenamine stock solution and distilled water with a few ml. of an M/5 borate buffer of pH 8.3–9.2 added. The more alkaline the solution, the faster it will work but the more likely it is to produce a brownish background, especially in the case of unduly long incubation. The reaction will show up in a yellowish-brown shade in about 1 hour. Inspect sections under the microscope once every 30 or 60 minutes; as soon as glycogen, mucin, etc., appear in black or in a very dark purplish-brown shade while the background is still a very light tobacco brown, remove sections from the silver solution, rinse in distilled water, and tone with a 0.1–0.2 per cent solution of gold chloride for about 10 minutes. This treatment usually completely bleaches the background. Remove unreacted silver by a short rinse with dilute (1–3 per cent) Na thiosulfate. Wash and counterstain as desired (e.g., with hematoxylin and eosin). Dehydrate and mount.

If the background is too dark, carefully differentiate the section (before gold toning) in a mixture containing about 0.2 per cent of ferric sulfate and 0.5 per cent of sulfuric acid

until the background is a pale yellow-brown. Overdifferentiation may weaken the reaction proper.

Oxidation by chromic acid followed by Schiff's reagent is Bauer's method;[33] oxidation by periodate followed by Schiff's reagent is the Hotchkiss-McManus method.[7, 35]

Etcheverry and Mancini[41] have described a method for carbohydrates based on the fact that tungstic acid, on exposure to ultraviolet light, is reduced by carbohydrates to a blue oxide of unknown composition. The mechanism of the reaction is not clear. The authors believe that it involves the oxidation of glycols to aldehydes.

The method is mentioned only as a curiosity; it cannot compete with other techniques which give a much better color contrast.

Specificity of aldehyde reactions after oxidative procedures.—As mentioned before, Schiff's reagent applied to paraffin or celloidin sections following the oxidative procedures described is specific for vicinal OH or OH and NH_2 groups, which occur only in carbohydrates and in a few amino acids. The same applies to the silver reagent after chromic acid, since substances which would reduce it without previous treatment (uric acid, phenols, melanin and its precursors) are destroyed by oxidation.

Two exceptions, however, must be made. First, under certain conditions, both normal and pathological, unsaturated fatty acids of the tissues may undergo changes resulting in the formation of peculiar substances the exact nature of which is not clearly understood. They exhibit the character of aldehydes and/or peroxides; most, but not all, of them are removed by the procedures of dehydration and embedding. They may be fairly well preserved in paraffin sections after prolonged fixation in bichromate-containing mixtures and give a positive Schiff reaction, to some extent even without

41. Etcheverry, M. A., and Mancini, R. E.: Rev. Soc. argent. de biol., **24**:156, 1948.

oxidative pretreatment (example: ceroid). Second, elastic fibers in certain locations (mainly in the inner elastic coat of small-caliber arteries) contain an unidentified nonlipid substance which behaves as an aldehyde whether or not an oxidative pretreatment is employed.

Differentiation between Schiff-positive substances.—Although normally the localization and morphology of glycogen is quite different from that of mucin, the problem of distinguishing between the two substances may come up once in a while. One of the oldest methods of differentiation is the saliva test. It is based on the fact that saliva contains a diastatic enzyme which will dissolve glycogen and starch but not mucin, amyloid and other related substances which may stain in a shade indistinguishable from that of glycogen. It may be appied to all staining methods, whether histochemical or not. Therefore, if a substance stains without pretreatment but fails to do so after an exposure to saliva of about 30–60 minutes, it must be glycogen or starch. On the other hand, if it persists in staining after the saliva test, it cannot be glycogen. At present, more appetizing and sanitary tests have replaced the time-honored saliva test. A number of highly active diastase (amylase) preparations are available which are specific and efficient. Being protein in nature, they will not diffuse through collodion; therefore, if they are to be used, sections should not be coated with collodion, and collodion should be removed from celloidin-embedded material. Lillie and Greco[42] recommend the use of a 1 per cent dilution of extract of malt, U.S.P., in a phosphate buffer of pH 6.8–7.4. A 1 per cent solution of malt or animal diastase (Nutritional Biochemicals) is even more effective. These enzymes will remove glycogen and starch from sections not over 8 μ thick within 15 minutes at room temperature. Prolonged exposure (24–48 hours) may remove all carbohydrate material.

A number of other specific enzymes for the hydrolysis of various mucopolysaccharides have become available recently

42. Lillie, R. D., and Greco, J.: Stain Technol., **22**:67, 1947.

(hyaluronidase, pectinase, polygalacturonidase, etc.).[43–45] Hyaluronidase appears to be specific in abolishing some or all staining reactions (depending on the source of the enzyme and on the length of exposure) due to hyaluronic acid and to chondroitin;[46] the limits of specificity of the other enzymes remain to be established.

B) *Methods for individual members of the group.*—

a) *Glycogen and starch.*—The oldest method for starch is the staining by iodine;[47] it is mentioned as early as 1825.[48] Glycogen will stain in a mahogany brown, native starch in dark blue. Iodine can be applied in the form of Lugol's solution,[49] iodine vapor, or a solution of iodine in paraffin oil.[50] The iodine reaction is not absolutely specific; amyloid, some protein substances, and lecithin[51] will also stain. Galactogen does not stain.[52] The degree of contrast obtained is not too high, and the preparations are not stable. Although some workers still use it extensively, the iodine reaction can be said to have little more than a historical interest.

Best's carmine.[34]—This is a purely empirical but highly selective stain. Besides glycogen, it may stain mast cell granules, mucin, and fibrin, but in so much lighter shades that confusion is unlikely.

43. Hale, C. W.: Nature, **157**:802, 1946.

44. Grishman, E.: Bull. Internat. A. M. Mus., **28**:104, 1948.

45. Gersh, I., and Catchpole, H. R.: Am. J. Anat., **85**:457, 1949; Stoughton, R., and Wells, G.: J. Invest. Dermat., **14**:37, 1950; McManus, J. F. A., and Saunders, J. C.: Science, **111**:204, 1950; McManus, J. F. A.: Am. J. Path., **26**:690, 1950; Bunting, H.: Ann. New York Acad. Sc., **52**:977, 1950.

46. Mathews, M. B., Roseman, S., and Dorfman, A.: J. Biol. Chem., **188**:327, 1951.

47. Mancini, R. E.: J. Nat. Cancer Inst., **10**:1371, 1950; Gage, S. H.: Tr. Am. Micr. Soc., **28**:203, 1906.

48. Caventou, J. B.: Ann. de chim. et de phys., **31**:337, 1826.

49. Langhans, T.: Virchows Arch. f. path. Anat., **120**:28, 1890.

50. Mancini, R. E., and Celani-Barry, R.: Rev. Soc. argent. de biol., **19**:493, 1943; Mancini, R. E.: Medicina, **7**:327, 1947; Mancini, R. E.: Anat. Rec., **101**:149, 1948.

51. Romieu, M.: Compt. rend. Soc. de biol., **96**:1230, 1927.

52. Grainger, J. N. R., and Shillitoe, A. J.: Stain Technol., **27**:81, 1952.

Method

Use celloidin sections or protect paraffin sections by dipping them into dilute (0.5–1 per cent) collodion in alcohol-ether between the first and second alcohols. Prepare the following stock solution:

Carmine	2 g.
Potassium carbonate	1 g.
Potassium chloride	5 g.
Distilled water	60 ml.

Simmer mixture gently for a few minutes; cool. Add 20 ml. of concentrated ammonia water. This solution will keep in the refrigerator for several months. For use, dilute 10 ml. with 15 ml. of concentrated ammonia water and 15. ml. of 95 per cent alcohol. This solution must be made fresh every time. Filter if not perfectly clear.

Stain nuclei rather dark with hematoxylin; rinse slide. Stain for 5–10 minutes in the dilute mixture. Differentiate in 60 per cent alcohol to which a few drops of ammonia water are added. Dehydrate and mount. Glycogen brilliant red.

b) *Mucin.*—

Mayer's[53] *mucicarmine stain.*—This is an empirical but specific stain for most types of mucus. Some varieties, however, are not stained; species and organ differences are quite marked. Mucus staining with mucicarmine also exhibits metachromatic properties, whereas mucicarmine-negative mucus is not metachromatic (see under "Metachromasia").

Method (modification of Southgate)[54]

Prepare the following stock solution: simmer gently a mixture of 1 g. of carmine, 0.5 g. of anhydrous aluminum chloride (or 0.9 g. of $AlCl_3 \cdot 6H_2O$), 1 g. of aluminum hydroxide, and 100 ml. of 50 per cent alcohol until it turns into a deep ruby-red liquid. This usually takes a few minutes. Let stand for 24 hours and filter; keep filtrate in the icebox.

53. Mayer, P.: Mitt. zool. Stat. Neapel, **12**:303, 1896.
54. Southgate, H. W.: J. Path. & Bact., **30**:729, 1927.

Fix tissues in alcohol or formalin-alcohol. Other fixatives are also usable, but the background may be stained more or less intensely. Stain nuclei with hematoxylin.

Rinse slide in water; stain in mucicarmine for about 15 minutes; rinse in water, dehydrate, and mount. Some kinds of mucus stain intensely red; others are much paler or may fail to stain altogether. If staining time is prolonged, hyaluronic acid and glycogen may become stained, although in a much paler shade.

The Metachromatic Staining of Acid Polysaccharides

The concept of metachromasia.—It was observed simultaneously by Cornil,[55] Jürgens,[56] and Heschl[57] in 1875 that methyl violet stains amyloid red and other tissue structures blue. This phenomenon—namely, the staining of certain tissue components in a color different from that of the dye solution itself—has been named "metachromasia" by Ehrlich.[58] According to his nomenclature, the shade of the dye solution is the orthochromatic shade; the different shade in which certain tissue elements stain is the metachromatic shade; the substances which exhibit the property of staining in the metachromatic shade are the chromotropic (or metachromatic) substances. It must be made clear that metachromasia applies only to the cases in which both shades are produced by a single dyestuff and not by a mixture of dyes. Some commercial dyes may contain substantial admixtures, as impurities, of other related dyestuffs.

The number of dyes exhibiting metachromatic properties is large. However, only a few of them have practical importance. The list includes thionin, the azures, toluidin blue, cresyl violet, and methyl violet. Methyl green, used for the metachromatic staining of amyloid, owes its metachromatic properties to its content of methyl violet. The most typical

55. Cornil, V.: Compt. rend. Acad. d. sc., **80**:1288, 1875.
56. Jürgens, R.: Virchows Arch. f. path. Anat., **65**:189, 1875.
57. Heschl, R.: Wien. med. Wchnschr., **25**:712, 1875.
58. Ehrlich, P.: Arch. f. mikr. Anat., **13**:263, 1877.

common property of these dyes is that their shade depends on the concentration of the solution (although this feature has not been investigated in the case of methyl violet). Dilute solutions are bluish, and, as the concentration is raised, the shade shifts progressively toward red. The absorption spectra of these dyes show two (or three) maxima. In the case of toluidin blue, for instance, one of the peaks (α) is around 630 mμ, the second one (β) around 590 mμ, and the third one (γ) around 560–70 mμ. In dilute solutions the α peak is the tallest; the other two are barely noticeable. With increasing dye concentration the β peak will become more and more prominent and actually surpass the α peak. The γ peak is observed only at rather high concentrations. Acidification, high temperature, and the addition of alcohol tend to suppress the β peak; alkalinization, low temperature, and the addition of chromotropic substances have the opposite effect.

The physicochemical nature of metachromasia is not completely elucidated. According to Lison, [59, 60] it is due to a tautomeric form (imino base) of the dye, which is in a labile equilibrium with the orthochromatic form. This equilibrium is displaced toward the metachromatic form by chromotropic substances. Michaelis and Granick,[61] on the other hand, have formulated the theory that the metachromatic form of the dye is simply a dimer (or polymer) of the orthochromatic (monomer) form. In the absorption spectrum, the absorption maximum of the monomer is represented by the α peak; that of the dimer by the β peak; that of polymers by the rather flat plateau of the γ peak. Factors favoring polymerization are high concentration, low temperature, relatively high pH values, an aqueous medium, and especially the presence of large molecules with suitably placed and spaced acidic groupings (–COOH, –OPO(OH)$_2$, –OSO$_2$OH) which can hold the polymer dye molecule together by its

59. Lison, L.: Acad. roy. Belgique, cl. de sc., **19**:1332, 1933.
60. Lison, L.: Arch. de biol., **46**:599, 1935.
61. Michaelis, L., and Granick, S.: J. Am. Chem. Soc., **67**:1212, 1945.

amino groups. Such substances with multiple acidic groupings are the chromotropic substances. Depending on the strength of the acidic groups, the degree of metachromasia they produce may vary considerably. Some types of metachromasia can exist only in an aqueous medium and at a neutral or slightly acid reaction (desoxyribose nucleic acid); others will resist acid down to pH 3 and mounting in glycerol (some connective-tissue polysaccharides). The extreme degree is resistant both to acid and to dehydration by acetone or alcohol, although the latter agents almost invariably decrease the color contrast. Lison[60, 62] has shown that the alcohol-resistant form of metachromasia ("true" metachromasia) is due to the presence of sulfuric esters of large molecular size. Until recently the only known substances occurring in animal tissues and exhibiting true metachromasia actually were such sulfuric esters (heparin, chondroitin-, and mucoitin-sulfuric acids). However, in 1940 Bignardi[63] showed that, if glycogen is treated with chromic acid until it becomes negative by the iodine stain and negative or weakly positive by Schiff's reaction (more than 3 hours' exposure to 4 per cent chromic acid), it will exhibit true metachromasia. Francini[64] was able to show the same change in the case of starch. Glycogen made metachromatic by long chromation also strains intensely with mucicarmine. The intestine of some species (e.g., the hedgehog) contains both mucous and mucoid cells (the latter stain poorly with mucicarmine and are not metachromatic). It is a highly interesting fact that, if sections of such a tissue are subjected to prolonged chromation, the mucinous cells will lose their typical staining properties while the mucoid cells become metachromatic and mucicarmine-positive.[65] The explanation of these findings is not

62. Lison, L.: Compt. rend. Soc. de biol., **118**:821, 1935.
63. Bignardi, C.: Atti Soc. ital. sc. nat., **79**:85, 1940.
64. Francini, E.: Nuovo gior. bot. ital., **47**:531, 1940.
65. Bignardi, C.: Boll. di zool., **10**:219, 1939; Bignardi, C.: Atti Soc. nat. e mat. Modena, **71**:59, 1940.

entirely clear, but it is reasonably safe to asume that the underlying chemical change is not the detachment or attachment, respectively, of sulfuric groups to a polysaccharide. It is much more likely that a maximum number of carboxylic groups favors metachromasia; these are present in some substances (mucin, amyloid, etc.) and are destroyed by prolonged chromation. They are absent in other substances (glycogen, starch, mucoid) but can be produced via the intermediate stage of aldehydes by the same oxidative treatment. In any case, overchromated glycogen appears to be an example of a nonsulfate polysaccharide exhibiting true metachromasia. The latest addition to the substances exhibiting true metachromasia is ribose nucleic acid. This substance was always considered to be nonmetachromatic (in Lison's sense); however, Flax and Himes[66] have shown that under certain conditions (specifications not clear) it will stain in a strongly metachromatic shade, resistant to alcohol.

Popper, György, and Goldblatt[67] described a peculiar metachromasia of ceroid stained with methyl green (not stated whether or not free from methyl violet). The nature of this metachromasia has not been investigated; it may be related to that of myelin as seen with the techniques of Feyrter[68] and Chang.[69] Wislocki and Singer[70] believe that Feyrter's metachromasia is due to the presence of sulfatides.

Metachromasia of the mucinoid substances, heparin and chondroitinsulfuric acid, on the one hand, and that of amyloid, on the other, are somewhat different, although, according to recent investigations, amyloid (not included in Meyer's classification) is a polysaccharide sulfate, closely similar to

66. Flax, M. H., and Himes, M. H.: Anat. Rec., **108**:529, 1950; Himes, M. H., and Flax, M. H.: Anat. Rec., **108**:539, 1950.

67. Popper, H., György, P., and Goldblatt, H.: Arch. Path., **37**:161, 1944.

68. Feyrter, F.: Virchows Arch. f. path. Anat., **296**:645, 1936; Feyrter, F.: Wien. klin. Wchnschr., **55**:461, 1942; Feyrter, F., and Pischinger, A.: Wien. klin. Wchnschr., **55**:463, 1942.

69. Min-Chueh Chang: Anat. Rec., **65**:437, 1938.

70. Wislocki, G. B., and Singer, M.: J. Comp. Neurol., **92**:71, 1950.

chondroitinsulfate.[71] While both the mucinoids and amyloid will stain metachromatically with all the dyes enumerated,[72, 73] the metachromasia of mucinoids with methyl violet is very much less marked than with the thiazin and oxazin dyes, and the opposite holds for amyloid. This fact shows that the phenomenon of metachromasia is more complex than it would appear from the relatively simple theory of Michaelis and Granick.[61]

The results of metachromatic staining of mucin vary greatly with the tissue, fixation, and other incalculable details of technique. Actually, these random and uncontrollable variations appear to be more important than the quality of the dye used or the special method followed. Of course, it cannot be denied that a certain batch of dye may give unusually good or unusually bad results, or that one of the specific techniques may yield better pictures than other methods in a given case. However, there is no single method which can be depended upon to give optimal results with all types of material. In our hands, satisfactory results were usually obtained with pinacyanol, introduced by Sylvén,[74] with toluidin blue and with celestin blue.[75] For the metachromatic staining of mucin, the use of almost any type of fixative, with the exception of bichromate-containing mixtures, is permissible.

Methods

a) *Toluidin blue or pinacyanol.*—Use a dilute (about 0.02–0.05 per cent) solution of either pinacyanol in distilled water or toluidin blue in a citrate buffer of pH 3.5–4.5. Stain section for 10–15 minutes or until, on inspection under the micro-

71. Hass, G.: Arch. Path., **34**:92, 1942.

72. Johansson, G. A., and Wahlgren, F.: Acta path. et microbiol. Scandinav., **15**:358, 1938.

73. Bignardi, C., and Casella, C.: Boll. Soc. med.-chir. Pavia, **55**:843, 1941.

74. Sylvén, B.: Personal communication.

75. Lendrum, A. C.: In Recent advances in clinical pathology (London: J. & A. Churchill, 1947), p. 457.

scope, nuclei are blue and mucin intensely pink. Rinse thoroughly in water, dehydrate rapidly in absolute acetone or alcohol, clear in xylene, and mount in balsam. Mucin, cartilage ground substance, and mast cells appear in shades of purple-blue to purple-red; nuclei, clear blue. Metachromasia of interfibrillar substance of connective tissue is greatly weakened by dehydration; this substance must be studied in unstable, water-mounted sections.

b) *Celestin blue.*—Dissolve 0.1 per cent celestin blue in a 5 per cent solution of ammonium (or potassium) aluminum sulfate. Stain sections in this solution for 2–3 hours. Rinse under the tap, dehydrate, and mount. The color contrast is less brilliant than with toluidin blue but much more resistant to alcohol.

Hale's technique for acid polysaccharides.—Hale described a technique[43] for the demonstration of acid polysaccharides, based upon the adsorption of colloidal iron hydroxide on acidic tissue components. In a second step the adsorbed iron is converted into Prussian blue.

While the method sometimes gives a most beautiful and selective staining of some types of mucin, it cannot by any means be considered a specific method for acid polysaccharides. Not even all types of mucin are stained by it, but only those which are metachromatic with toluidin blue; the nonmetachromatic mucus of the stomach and of Brunner's glands is left entirely unstained. According to Grishman,[44] even some types of metachromatic mucin are negative with Hale's stain. Mast cells, which contain a strongly acid polysaccharide, also stain very poorly. For connective-tissue polysaccharides, Hale's method is greatly inferior to the McManus stain in respect to both sharpness and uniformity of results, especially after fixations other than alcohol. It usually stains chromatin quite intensely; in addition, there is a diffuse light-blue tinge to the background.

In summary, the specificity of Hale's method is not limited to any chemically defined substance. The method will be de-

scribed here mainly because it is useful for the demonstration of some types of mucin.

Method

Prepare a colloidal solution of ferric hydroxide by the method of Rinehart and Abul-Haj.[76] Dissolve 30 g. of ferric chloride (lumps) in 100 ml. of distilled water, add 40 ml. of glycerol and, in small portions and under continuous stirring, 22 ml. of concentrated ammonia water. Dialyze the mixture under the tap for about 48 hours (*caution:* fill the dialyzing bag less than halfway because there will be about a 2.5-fold increase in volume). Filter the dialyzate; it will keep indefinitely. For use, mix about 10 parts of the dialyzate and 1 part of concentrated acetic acid. This mixture is stable for 2 days only. Stain slides in the mixture for about 10 minutes, rinse them in repeated changes of distilled water, and immerse for 10 minutes in a fresh solution containing about 1 per cent each of hydrochloric acid and potassium ferrocyanide. Counterstain with a red nuclear dye, dehydrate, and mount.

The mannitol-$FeCl_3$ method of Lillie and Mowry[77] gives results almost identical with those of Hale's technique.

Ritter and Oleson[78] suggest the McManus stain as a counterstain. Some substances will appear in a blue, others in a red shade; the interpretation of this difference is not clear.

c) *Heparin* (*granules of mast cells*).—Holmgren and Wilander[79] suggest primary fixation in a 4 per cent solution of basic lead acetate for 12–24 hours; this may be followed, for better cytological detail, by formalin. Lead acetate precipitates heparin in the form of an insoluble lead salt. After this

76. Rinehart, J. F., and Abul-Haj, S. K.: Arch. Path., **52**:189, 1951.

77. Lillie, R. D., and Mowry, R. W.: Bull. Internat. A. M. Mus., **30**:91, 1949.

78. Ritter, H. B., and Oleson, J. J.: Am. J. Path., **26**:639, 1950.

79. Holmgren, H., and Wilander, O.: Ztschr. f. mikr.-anat. Forsch., **42**:242, 1937; Holmgren, H.: Ztschr. f. wissensch. Mikr., **55**:419, 1938; Holmgren, H.: Ztschr. f. mikr.-anat. Forsch., **47**:489, 1940.

fixation, the mast cells will stain almost blue-black, with only a slight tinge of red. The shade is probably due, at least in part, to the adsorption of the dye on the lead compound (toluidin blue is adsorbed strongly on various insoluble lead precipitates).

d) *Amyloid.*—The term "amyloid" was coined by Virchow[80] to denote a homogeneous, somewhat translucent substance which is deposited in connective tissue under certain pathologic conditions and is stained somewhat like starch (*amylos* equals starch) by iodine.

Chemically, the characteristic component of amyloid appears to be a sulfuric ester of a polysaccharide.[72]

Depending on their age and other poorly understood factors, the staining reactions of deposits of amyloid are rather variable. Recent deposits may not show some or even any of the typical staining properties, whereas old deposits usually stain according to textbook specifications.

The two most important tinctorial features of amyloid are its stainability by iodine and its metachromasia.

The iodine reaction.—This is very similar to that of glycogen, with the exception that the mahogany brown shade may turn into a dark gray-bluish or greenish one on the application of dilute mineral acid.

Metachromasia of amyloid: Methods

1. Stain section in a dilute (0.1–0.2 per cent) solution of methyl violet (or crystal violet) for about 10 minutes. Differentiate in 1 per cent acetic acid until amyloid is purple-red and the background blue. Rinse, mount in glycerin jelly. Preparations not permanent.

2. This variant yields permanent preparations. Float the paraffin sections directly on the dye solution (warmed to about 37°–45°C.) for about 15–20 minutes. Refloat them on water to wash out excess dye, then on 1–2 per cent acetic

80. Virchow, R.: Virchows Arch. f. path. Anat., **6**:416, 1854.

acid until sections are sufficiently differentiated. Refloat on water once more; mount on slides. Dry slides, remove paraffin with xylene, and mount section in balsam. This technique avoids all dehydrating agents and preserves metachromasia very well.

II. NUCLEIC ACIDS

The three components of the nucleic acids are (1) purine and pyrimidine bases; (2) pentose or desoxypentose sugar; and (3) phosphoric acid. The histochemical methods for their demonstration will accordingly be divided into three groups, depending on which of the components is identified. The specificity of the identifying reactions can be checked by extraction techniques, enzymatic or other, which remove nucleic acids in a selective way.

1. The purine and pyrimidine bases can be demonstrated by the ultraviolet absorption method only. Recently, Danielli[81] has proposed another method which may be called descriptively "double azo-coupling." It is based on the theory that purine and pyrimidine bases will couple with diazonium salts. The resulting azo dye is, however, only pale yellowish in shade and unsuitable for direct observation. For this reason, Danielli uses a tetrazonium compound (of benzidine or dianisidine) which attaches itself to the base by only one of its diazo groups; the other free, diazonium group can then be coupled with highly chromogenic naphthol, and an intensely colored (purplish) azo dye results. Tyrosine, histidine, and tryptophane give similar color reactions; benzoylation, however, will abolish their coupling ability while it will leave that of the purine and pyrimidine bases intact.

The chemical basis of this method does not appear to be sufficiently firm.

First of all, chemical studies on the azo-coupling of purines

81. Danielli, J. F.: Symp. Soc. Exper. Biol., **1**:101, 1947.

(Burian,[82] Johnson and Clapp,[83] Steudel,[84] H. Fischer,[85] and Hunter[86]) all agree that this coupling takes place exclusively in the presence of a high concentration of caustic alkali (carbonates, e.g., will not do). It is also specified that purines substituted in the imidazole ring (caffeine, theobromine, nucleotides) will not react at all.[82, 85] Therefore, if azo-coupling in slides is attempted according to the suggestion of Mitchell,[87] as modified by Danielli[81] (at pH 9), no reaction can be expected to take place. Actually, in Coujard slides, carrying marks made with gelatin alone and with suspensions of guanine, adenine, xanthine, uracil, uric acid, and RNA, no differentiation of any kind can be obtained.

Second, all diazonium compounds are quite labile, especially at an alkaline reaction. On standing (even at 0° C.), they form dark-colored decomposition products which will stain almost anything (e.g., a strip of filter paper or even a collodion membrane) and cannot be washed out completely. When pieces of such stained material, after thorough washing in water and alcohol, are immersed in an alkaline solution of β-naphthol, they will stain intensely purple. Tissues treated by this method are stained quite diffusely, without much differentiation of morphologic detail. The enterochromaffin granules are the only exception to this. They stain selectively and quite intensely both after the primary and after the secondary azo-coupling (the latter first recommended by Clara and Canal in 1932).[88] There can be no doubt that after acylation there is a marked decrease in the staining of all tissue structures, but the contrast is not much improved. It is remarkable that reactivity of the entero-

82. Burian, R.: Ztschr. f. physiol. Chem., **51**:425, 1907.
83. Johnson, T. B., and Clapp, S. H.: J. Biol. Chem., **5**:163, 1908.
84. Steudel, H.: Ztschr. f. physiol. Chem., **48**:425, 1906.
85. Fischer, H.: Ztschr. f. physiol. Chem., **60**:69, 1909.
86. Hunter, G.: Biochem. J., **30**:745, 1936.
87. Mitchell, J. S.: Brit. J. Exper. Path., **23**:296, 1942.
88. Clara, M., and Canal, F.: Ztschr. f. Zellforsch. u. mikr. Anat., **15**:801, 1932.

chromaffin phenol is not completely abolished even after 24 hours' acylation.

For the reasons mentioned, great caution is warranted in the evaluation of the results of Danielli's technique. Its mechanism is not entirely clear, and the possibility that the results are largely nonspecific cannot be ruled out.

2. The sugar moiety is identified by aldehyde reactions after acid hydrolysis. The most commonly used aldehyde reagent is Schiff's, which will demonstrate only desoxyribose (Feulgen's nucleal reaction).[89] Schiff's reagent can be replaced by other chromogenic carbonyl reagents, such as 2-hydroxy-3-naphthoic acid hydrazide followed by azo-coupling,[90] with essentially similar results. Turchini's reagent (9-phenyl-2,3,7-trihydroxy-6-fluorone) will condense with both ribose and desoxyribose and, in fact, even with hexoses.[91] The condensation products with these sugars have different colors (ribose, yellowish pink; desoxyribose, bluish purple; hexoses, reddish purple). RNA must be protected by fixation in dichromate-containing mixtures (which make nucleic acids more resistant to hydrolysis; Hillary);[92] otherwise it may be lost in the procedure. No reports on the reliability of this method are available so far except by Turchini and his co-workers.

89. Feulgen, R.: Die Nuclealfärbung. In Abderhalden's Handb. d. biol. Arbeitsmeth., V²-**2**:1054, 1932.

90. Pearse, A. G. Everson: J. Clin. Path., **4**:1, 1951.

91. Turchini, J., Castel, P., and Kien, K.: Trav. soc. chim. biol., **35**:1329, 1943; Turchini, J., Castel, P., and Kien, K.: Montpellier méd., **23–24**:599, 1943; Turchini, J., Castel, P., and Kien, K.: *ibid.*, **25–26**:396, 1944; Turchini, J., Castel, P., and Kien, K.: Bull. d'histol. appliq. à la physiol., **21**:124, 1944; Turchini, J., and Gosselin de Beaumont, L. A.: Compt. rend. Soc. de biol., **139**:584, 1945; Kien, K., and Sentein, P.: Compt. rend. A. anat., p. 264, 1947; Turchini, J., Castel, P., and Kien, K.: Compt. rend. A. anat., p. 456, 1947; Turchini, J., and Kien, K.: Compt. rend. A. anat., p. 391, 1948; Turchini, J., Castel, P., and Kien, K.: Compt. rend. Soc. de biol., **142**:1277, 1948; Turchini, J.: Exper. Cell Research, Suppl., **1**:105, 1949; Turchini, J., and Kien, K.: XIII^{e} Congrès internat. de zool. Paris, p. 207, 1949.

92. Hillary, B. B.: Bot. Gaz., **101**:276, 1939.

The specificity of the Feulgen nucleal reaction has been the subject of a lively controversy until very recently. The following arguments have been voiced against it:

1. There need be no chemical interaction between nucleic acid and the reagent. The active dye may be fuchsin itself, adsorbed by the nuclei, just as, e.g., alumina can adsorb fuchsin from Schiff's reagent. The effect of hydrolysis may consist simply in a dissolution of the cytoplasm while the nucleus is relatively resistant; in this way the contrast between the nucleus and the cytoplasm is enhanced. The fact that an excess of bisulfite (which should block aldehyde groups) does not prevent staining of the nuclei shows that it is not due to an aldehyde reaction.[93]

2. Recolorization of Schiff's reagent may be due to nonaldehydic substances; pyridine and various purine bases will give color reactions more or less readily.[94]

3. It is admitted that the dye produced by the interaction of Schiff's reagent and hydrolyzed DNA is a true aldehyde addition product; however, it is soluble in water and a good stain for chromosomes,[95] especially for chromosomin and histone.[96] Therefore, DNA only contributes to the dye which then stains something else.[91, 97]

The weak points of these arguments can be easily shown:

1. The specificity of the nuclear staining is not a matter of increase in contrast; without hydrolysis nothing whatsoever will stain in a properly performed Feulgen test. As for the blocking of aldehyde groups by an excess of SO_2, it must be remembered that aldehyde-bisulfite addition compounds (especially those of aldehydes of higher molecular weight) are very unstable and prone to break down.[98]

2. Recolorization of fuchsin by nonaldehydic compounds

93. Carr, J. G.: Nature, **156**:143, 1945.
94. Semmens, C. S.: Nature, **146**:130, 1940.
95. Choudhuri, H. C.: Nature, **152**:475, 1943.
96. Sibatani, A.: Nature, **166**:355, 1950.
97. Stedman, E., and Stedman, E.: Symp. Soc. Exper. Biol., **1**:232, 1947.
98. Dodson, E. O.: Stain Technol., **21**:103, 1946.

is a pseudo-reaction, not resistant to acid and alcohol. Also, the recolorization of Schiff's reagent by purines could not be confirmed.[99] That the active dye is really an aldehyde addition product can be proved by blocking the reaction with specific carbonyl reagents.[100] Cyanide is especially effective.[101] The successful substitution of Schiff's reagent by other reagents has been mentioned. The aldehyde compound must come from DNA; if the latter is removed by specific enzymes, the Feulgen reaction is abolished.

3. The dye prepared in the test tube from hydrolyzed DNA and Schiff's reagent is soluble (although not dialyzable).[102] However, this does not apply to tissue sections in which nucleic acid is present in firm chemical union with proteins and in a completely insoluble state. It is not made soluble by mild acid hydrolysis.[103] The normal HCl from a Coplin jar in which nine slides, carrying several large tissue sections each, were hydrolyzed for 12 minutes did not show the slightest trace of color with Schiff's reagent. There is no reason to assume that a degraded but insoluble DNA would form a soluble addition compound with Schiff's reagent; in fact, there is evidence to the contrary.[104] In Coujard slides, marks made with DNA of high purity and fixed in formalin-alcohol gave an intense and sharp reaction, with absolutely no indication of diffusion; marks made with RNA and several proteins were completely negative. Of course, it is conceivable that traces of moderately soluble fragments may be liberated during hydrolysis and converted into dye which may stain structures in the immediate proximity. However, it is difficult to explain why the infinitesimal concentration of dye

99. Barber, H. N., and Price, J. R.: Nature, **146**:335, 1940.

100. Lessler, M. A., and Kopac, M. J.: Anat. Rec., **108**:531, 1950; Lessler, M. A., and Kopac, M. J.: *ibid.*, **108**:578, 1950; Lhotka, J. F., and Davenport, H. A.: Stain Technol., **26**:35, 1951.

101. Gomori, G.: Unpublished.

102. Ely, J. O., and Ross, M. H.: Anat. Rec., **104**:103, 1949.

103. Brachet, J.: Experientia, **2**:142, 1946.

104. Li, C., and Stacey, M.: Nature, **163**:538, 1949.

present locally at any given moment (both the hydrolyzed DNA and the dyestuff are assumed to be soluble and mobile) should stain the chromosomes intensely and sharply in a matter of a few minutes when a strong solution of the dye prepared in the test tube from hydrolyzed DNA and Schiff's reagent will give only a blurred and weak staining, even on prolonged application.

Additional proofs for the specificity of the Feulgen stain and of the correctness of its localization are the staining of isolated chromatin threads[105] and the excellent agreement between the Feulgen reaction and the results of localizing ultraviolet spectrophotometry (Caspersson).[106]

In summary, it may be said that the Feulgen reaction is a reliable method for the specific localization of DNA, provided that adequate unhydrolyzed and hydrolyzed but aldehyde-blocked controls are used in the case of doubt. The nature of the positive Feulgen reaction in nervous elements (in unembedded tissue only), reported by Liang[107] and Chu,[108] is not clear.

The Feulgen reaction can be performed in tissue blocks;[109] however, the section technique is much more preferable.

The optimal duration of hydrolysis depends on the type of fixation. In the case of dichromate-free fixatives, the most intense reactions are obtained between 8 and 12 minutes (N HCl, 60°C.). Overhydrolysis will gradually abolish the reaction. There is a difference between the resistance of nuclei of various tissues, thymus nuclei being the most resistant (up to 30 minutes).[110] After dichromate-containing

105. Claude, A., and Potter, J. S.: J. Exper. Med., **77**:345, 1943; Barber, H. N., and Callan, H. G.: Nature, **153**:109, 1944.

106. Caspersson, T.: Nature, **153**:499, 1944.

107. Liang, H. M.: Anat. Rec., **99**:511, 1947.

108. Chu, C. H. U.: Science, **106**:70, 1947.

109. Lhotka, J. F., and Davenport, H. A.: Stain Technol., **22**:139, 1947; Lhotka, J. F., and Davenport, H. A.: *ibid.*, **24**:127, 1949.

110. DeLamater, E. D., Mescon, H., and Barger, J. D.: J. Invest. Dermat., **14**:133, 1950.

fixatives the optimum duration of hydrolysis is much less critical (10–30 minutes).[92]

Schiff's reagent itself may cause some hydrolysis on long exposure.[111] This is especially important in the case of staining unhydrolyzed control sections because of the possibility of obtaining false positive reactions.

The following procedure is recommended:

Method

Hydrolyze tissue sections in N HCl preheated to 58°–62°C. for 8–10 minutes. Wash them under the tap and stain in Schiff's reagent as described in the section on polysaccharides (p. 59). Naturally, omit counterstaining with hematoxylin; a yellow or green acid counterstain (picric acid, orange G, or light green) is permissible and may actually be advantageous.

3. The acid moiety. There are a few methods published for the specific identification of phosphoric acid in the nucleotides.[112] The reliability of these methods, especially as far as correct localization is concerned, is questionable (see under "Phosphorus").

Other methods demonstrate simply the presence of acidic groups of any kind. The use of indicators cannot be depended upon to yield accurate information because the color contrasts are not sharp enough.

The only methods to be used extensively are based on basophilia, the affinity for basic dyes, which is a property of any substance possessing acidic groups. To obtain confirmatory evidence for nucleotides as the cause of basophilia, specific extraction procedures must be resorted to in many cases because of the presence of basophilic substances other than nucleic acids (mucopolysaccharides) in the tissues.

The basic dyes most often used are methyl green, meth-

111. Serra, J. A.: Bol. Soc. Broteriana, **17**:203, 1943.

112. Lilienfeld, L., and Monti, A.: Ztschr. f. wissensch. Mikr., **9**:332, 1892; Serra, J. A., and Queiroz Lopes, A.: Port. acta biol., **1**:111, 1945.

ylene blue, thionin, toluidin blue, pyronin, and safranin. Methyl green stands in a class by itself on account of its unique property of staining some, but not all, of the basophilic substances. It will stain high-polymer DNA as it occurs in the nuclei or as obtained by gentle extraction procedures, and also sulfate-polysaccharides, but not depolymerized DNA or RNA in any form. The reasons for this curious behavior are not well understood.[113, 114] In practice, however, a combination of methyl green and pyronin (Pappenheim and Unna)[115] is a most useful dye mixture, giving an excellent contrast between green-staining DNA of nuclei and red-staining RNA. All other dyes mentioned will stain both the nucleotides and also acid polysaccharides; the latter may be stained in a metachromatic shade. It is important to use the dyes at a neutral or slightly acid (pH 5–6) reaction, because otherwise they may be taken up even by nonbasophilic structures.

The nature of the binding of basic dyes by basophilic substances is not completely understood. There are good indications that in a solution there is a strict stoichiometric relationship between the acidic groups and the amount of dye bound.[116] However, it is very questionable whether this relationship holds for the insoluble nucleoproteins of the tissues where an undetermined proportion of the acidic groups may be tied down to strongly basic proteins in a stable, nonreactive form. It is quite likely that in sections ionic forces, im-

113. Pollister, A. W., and Leuchtenberger, C.: Proc. Nat. Acad. Sc., **35**:111, 1949.

114. Vendrely, C., and Vendrely, R.: Compt. rend. Soc. de biol., **143**:1388, 1949; Kurnick, N. B.: J. Gen. Physiol., **33**:243, 1950; Kurnick, N. B., and Mirsky, A. E.: J. Gen. Physiol., **33**:265, 1950; Kurnick, N. B.: J. Nat. Cancer Inst., **10**:1345, 1950; Taft, E. B.: Exper. Cell Research, **2**:312, 1951.

115. Unna, P.: Plasmazellen. In Enzykl. mikr. Technik (2d ed.; 1910), p. 744.

116. Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A.: J. Biol. Chem., **72**:707, 1927.

portant as they may be, are not the only factors at work.[117] Other structural properties (the presence of polar groups; steric configuration), both of the adsorbing nucleoprotein and of the adsorbed dye, may be more important than acid-base affinity, just as in the case of cation exchangers[118] (resins and other substances). For instance, a compound as little acidic as silica (pK of silicic acid, ±9.7) will quantitatively remove methylene blue from a solution in 0.1 N sulfuric acid.

Many attempts have been made in the past to stain tissue sections with basic dyes at graded pH levels and to draw meaningful conclusions from the more or less abrupt changes in staining intensity.[119–29] The pH at which such sudden changes occur has variously been called the isoelectric point of the substance stained[119, 120, 122, 123, 126] or the acidic dissociation constant of its basophilic group.[124, 125] The fundamental assumptions behind such experiments are that (*a*) acidic substances will stain with increasing intensity above their isoelectric point (or pK of their acidic groups), while below this point their staining intensity will decrease rapidly; and

117. Michaelis, L.: Arch. f. mikr. Anat., **94**:580, 1920.

118. Whitehorn, J. C.: J. Biol. Chem., **56**:751, 1923; Walton, H. F.: Ion exchange equilibria. In Ion exchange, theory, and application, ed. F. C. Nachod (New York: Academic Press, 1949).

119. Pischinger, A.: Ztschr. f. Zellforsch. u. mikr. Anat., **3**:169, 1925–26.

120. Pischinger, A.: *ibid.*, **5**:347, 1927.

121. Hammarsten, E., Hammarsten, G., and Teorell, T.: Acta med. Scandinav., **68**:219, 1928; Zeiger, K.: Ztschr. f. Zellforsch. u. mikr. Anat., **10**:481, 1930.

122. Fautrez, J.: Bull. d'histol. appliq. à la physiol., **13**:202, 1936.

123. Levine, N. D.: Stain Technol., **15**:91, 1940.

124. Kelley, E. G.: J. Biol. Chem., **127**:55, 1939.

125. Kelley, E. G.: *ibid.*, **127**:73, 1939.

126. Seki, M., and Kohashi, Y.: Okajima's Folia anat. Jap., **19**:47, 1940.

127. McCalla, T. M.: Stain Technol., **16**:27, 1941.

128. Hydén, H.: Acta physiol. Scandinav., Vol. **6**, Suppl. 17, 1943.

129. Dempsey, E. W., Bunting, H., Singer, M., and Wislocki, G. B.: Anat. Rec., **98**:417, 1947; Singer, M., and Morrison, P. R.: J. Biol. Chem., **175**:133, 1948.

(*b*) the part of dye which is adsorbed in excess of the ion exchange mechanism can be removed with alcohol,[130] or that adsorption can actually be prevented by the use of detergents incorporated in the staining solution.[128]

Extreme caution is warranted in the interpretation of the results of such experiments. First of all, the results depend within wide limits on minor variations in technique (the dye used, its concentration, time of staining, washing, and differentiation). Especially differentiation is a very delicate step. While acid in an aqueous medium has a relatively moderate effect on the staining by basic dyes, traces of it transferred to the differentiating alcohol (either from the dye solution or from the buffer used to wash out the excess dye) may decolorize the section extensively during the first few seconds of contact, even at pH values as high as 4–4.5. On the other hand, tissues stained at a pH as low as 1.2 or lower (and they do stain with considerable intensity; even Gabbett's[131] methylene blue, which contains 25 per cent sulfuric acid, will stain nuclei), differentiated in a buffer of the same pH, and subsequently washed thoroughly to remove all traces of acid may retain a beautiful nuclear staining even after prolonged differentiation in alcohol.[132]

In summary, it may be said that, other things being equal, substances possessing acidic groups are more likely to stain with basic dyes, especially at pH levels below neutrality, than substances without such groups. However, basophilia is the result of many factors, most of which are very poorly understood. It does not permit the drawing of even approximately quantitative conclusions as to isoelectric points or the values of dissociation constants.

Extraction procedures

Strictly speaking, "extraction" is a misnomer if applied to the whole group of procedures to be described here, because

130. Michaelis, L.: Cold Spring Harbor Symp. Quant. Biol., **12**:131, 1947.
131. Gabbett, H. S.: Lancet, 1887-I, p. 757.
132. Gomori, G.: Unpublished.

some of them do not actually extract nucleotides but rather alter them sufficiently to abolish their normal staining characteristics. Most of the others, however, do break them down to small, soluble fragments which will diffuse out from the section.

The efficiency of all extraction procedures greatly depends on the type of fixation. Alcohol-, formalin-, and dichromate-fixed tissues are attacked with increasing difficulty in the order mentioned. Resistance to extraction also varies with the type of tissue. The procedures will be divided in two groups: nonenzymatic and enzymatic.

a) *Nonenzymatic procedures.*—Hot water will abolish the staining of DNA by methyl green, while the Feulgen reaction remains unchanged.[113] Exposure to 5 per cent citric acid at room temperature for 12 hours has the same effect.[133] According to Mirsky and Pollister,[134] nucleoproteins can be extracted from tissues with M NaCl; the use of this method has not been attempted histochemically.

Strong acids extract RNA first, and, on longer exposure, DNA is also removed. For instance, the Feulgen type of hydrolysis (N HCl; 60° C.) removes RNA in about 3–10 minutes;[135] DNA is not completely removed up to 50 minutes.[102] Five to 10 per cent perchloric acid removes RNA in 4–18 hours at 5° C. and in about 2 hours at 25° C.[136] DNA is not completely extracted at the latter temperature in 22 hours; at 70° C. both DNA and RNA are extracted in 20 minutes.[137] Five per cent trichloroacetic acid also extracts both nucleo-

133. Brachet, J.: Arch. de biol., **53**:207, 1942.

134. Mirsky, A. E., and Pollister, A. W.: Proc. Nat. Acad. Sc., **28**:344, 1942.

135. Deane, H. W.: Am. J. Anat., **78**:227, 1946; Vendrely, R., and Lipardy, J.: Compt. rend. Acad. sc., **223**:342, 1946; Vendrely-Randavel, C.: Compt. rend. Soc. de biol., **143**:294, 1949.

136. Ogur, M., and Rosen, G.: Fed. Proc., **8**:234, 1949.

137. Erickson, R. O., Sax, K. B., and Ogur, M.: Science, **110**:472, 1949; Seshachar, B. R., and Flick, E. W.: Science, **110**:659, 1949; Sulkin, N. M., and Kuntz, A.: Proc. Soc. Exper. Biol. & Med., **73**:413, 1950; Koenig, H.: J. Nat. Cancer Inst., **10**:1346, 1950.

tides at about the same rate.[138] Extraction of RNA with 0.1 N KOH is recommended by Sulkin.[139]

Henry and Stacey[140] have presented excellent evidence to show that the Gram-positive staining of bacteria is due to an RNA-Mg complex, extractable with a 2 per cent bile salt solution at 60° C. This bacterial RNA is obviously different from the ordinary type of RNA, which is Gram-negative and insoluble in bile salt solutions.

b) *Enzymatic procedures.*—Enzymatic hydrolysis of nucleic acids follows a course different from that seen in acid hydrolysis. The first stage is depolymerization of a variable degree; the next stage is the removal of PO_4 groups; and the last one is the breakdown of glycosidic linkages, which are the first ones to be attacked by acids.[141] It is very likely that every stage has its own enzyme or enzymes (depolymerase, phosphatase, nucleosidase) and that many of the enzyme preparations used in histochemistry are mixtures of several components. In fact, they are often contaminated by proteases of various kinds. This explains the different results obtained by the use of enzymes prepared in different ways.

The depolymerases of RNA and DNA are definitely specific; a good preparation of desoxyribonuclease will not attack RNA and vice versa.

According to Danielli,[81] the specificity of the nucleases is open to doubt. He also questions whether the histochemical application of enzymes can ever yield quantitative results, since the penetration of the large enzyme molecules into the interior of the section would be blocked by even a monolayer of protein. However, there is ample experimental evidence

138. Koenig, H., and Stahlecker, H.: J. Nat. Cancer Inst., **12**:237, 1951; Schneider, W. C.: J. Biol. Chem., **161**:293, 1945.

139. Sulkin, N. M.: Proc. Soc. Exper. Biol. & Med., **78**:32, 1951.

140. Henry, H., and Stacey, M.: Nature, **151**:671, 1943; Henry, H., Stacey, M., and Teece, E. G.: Nature, **156**:720, 1945; Henry, H., and Stacey, M.: Proc. Roy. Soc. London, B, **133**:391, 1946.

141. Catcheside, D. G., and Holmes, B.: Symp. Soc. Exper. Biol., **1**:225, 1947.

both for the specificity of at least some of the nucleases and for the quantitative effect of enzymes (for instance, diastase and ribonuclease).

The preparation of highly active ribonuclease of excellent specificity is simple and will be given here (Brachet's method).[142] The step of boiling the crude enzyme solution destroys all enzymes except the heat-resistant ribonuclease.

Method

Grind beef pancreas to a smooth pulp and suspend it for 24 hours at 37° C. in 1–2 volumes of 0.1 N acetic acid. Boil it for 10 minutes and filter. Neutralize the filtrate to about pH 6.9–7.5 and filter it once more. This solution can be kept in the icebox, with some camphor or thymol added, for several months. Brachet recommends dialysis as a last step, but it is not necessary.

Incubate sections in the enzyme solution for 1 hour at 65°–70° C. Treat a control section with a buffer of the same pH. The enzyme-treated section will show a complete loss of all basophilia due to RNA[143] (but not of that due to mucopolysaccharides). The Feulgen reaction of the nuclei is not affected.

Of the desoxyribonuclease preparations, McCarty's[144] appears to be the most specific. Its preparation is not easy; in most cases it will be simpler to purchase a commercial preparation. Desoxyribonuclease is not resistant to heat; it should be used around 37° C. A good preparation will abolish the Feulgen reaction of the nuclei but leave cytoplasmic basophilia intact.

According to Mazia,[145] intestinal phosphatase is also effective in removing RNA. Acid phosphatase has not been used histochemically.

142. Brachet, J.: Compt. rend. Soc. de biol., **133**:88, 1940.

143. Brachet, J., and Shaver, J. R.: Stain Technol., **23**:177, 1948; Deane, H. W.: Am. J. Anat., **78**:227, 1946.

144. McCarty, M.: J. Gen. Physiol., **29**:123, 1946.

145. Mazia, D.: Cold Spring Harbor Symp. Quant. Biol., **9**:40, 1941.

APPENDIX

Ascorbic Acid

Ascorbic acid has the unique property of reducing an acidified solution of silver nitrate almost instantly. This unusual reducing power can be utilized for its histochemical identification. The first histochemical experiments were made by Szent-Györgyi[146] even before the chemical nature of ascorbic acid was known.

While the specificity of the reaction (if performed correctly) is reasonably certain, the correctness of localization is much less so. Ascorbic acid is a highly diffusible substance which cannot be expected to stay at its original sites unless the tissue is frozen-dried. Its displacement cannot be avoided even by fixation in formaldehyde vapor[147] or by perfusing the tissue with reagent,[148] although localization (in the gross histological, but not cytological, sense) may be improved by these maneuvers. The experiments of Barnett and Fisher[149] clearly show the importance of physical factors, such as lipid-water interfaces, on the localization of the reaction.

According to Huszák,[150] Giroud and Leblond,[151] and Barnett and Bourne,[152] a negative reaction does not rule out the presence of ascorbic acid because some tissues seem to contain a substance which prevents the reduction of silver nitrate. The last-named authors believe that this inhibitor is glutathion. This substance actually slows down the reaction considerably in test-tube experiments.

Method[153]

The reagent is an aqueous or alcoholic 1–5 per cent solution of silver nitrate acidified with some acetic acid; concen-

146. Szent-Györgyi, A.: Biochem. J., **22**:1387, 1928.
147. Bourne, G.: Australian J. Exper. Biol., **11**:261, 1933.
148. Giroud, A., and Leblond, C. P.: Arch. d'anat. micr., **30**:105, 1934.
149. Barnett, S. A., and Fisher, R. B.: J. Exper. Biol., **20**:14, 1944.
150. Huszák, S.: Ztschr. f. physiol. Chem., **222**:229, 1933.
151. Giroud, A., and Leblond, C. P.: Nature, **138**:247, 1936.
152. Barnett, S. A., and Bourne, G.: J. Anat., **75**:251, 1941.
153. Giroud, A., and Leblond, C. P.: Bull. d'histol. appliq. à la physiol., **11**:375, 1934.

trations are of little importance. Small pieces of tissue are fixed directly in this fluid. In case of intravascular injection it is advisable to perfuse the tissue with an isotonic (about 5 per cent) solution of glucose first, because otherwise silver chloride and silver protein precipitates may clog the vessels. The reagent is allowed to act for not much longer than necessary for complete penetration of the tissue (5–20 minutes, depending on its size); the excess of silver is removed by repeated changes of 1 per cent Na thiosulfate, and the latter by distilled water. The tissue may be frozen-cut or dehydrated and embedded. A black granular precipitate indicates the presence of ascorbic acid. Unless exposure to the silver solution is unduly prolonged (in which case urates may react), false positive reactions need not be feared.

Gold chloride[147] can be used instead of silver nitrate; the results are claimed to be identical.

To the writer's knowledge, the demonstration of ascorbic acid has not been tried in sections of frozen-dried material. It is not even certain that the reduction of silver nitrate is prompt enough for a sharp localization. However, in attempts at using frozen-dried tissues it should be borne in mind that ascorbic acid is soluble in alcohol; therefore, the reagent should be applied to deparaffinized, air-dried sections.

B. LIPIDS

"Lipids" (synonyms: "lipoids," "fatty substances") is a term which will be used to denote a large group of miscellaneous chemical substances classified together, for histochemical purposes only, by their solubility properties. These properties are insolubility in water and solubility in several or all of the so-called "fat solvents" (alcohol, ether, chloroform, benzene, pyridine, acetone, etc.).

The histochemistry of lipids has been reviewed very thoroughly by Cain,[1] and the reader interested in the topic is urged to consult this excellent paper for detailed information.

An immense variety of lipid substances occurs in the ani-

1. Cain, A. J.: Biol. Rev. Cambridge Phil. Soc., **25**:73, 1950.

mal and plant kingdoms. The classification which follows does not claim completeness; it includes only such lipids as are likely to be met with in normal and pathological tissues of man and of the common laboratory animals.

Classification of Lipids

A. Paraffins (petrolatum).
B. Isoprene derivatives (carotenoids). This group includes carotene, vitamin A, visual purple, and some of the pigments of crustaceans.
C. Fatty acids and their derivatives.
   a) Fatty acids.
   b) Soaps, especially those of Ca. Although soaps are insoluble in fat solvents, they are included in this group for reasons of close chemical relationship.
   c) Triglycerids (neutral fats).
   d) Waxes (long-chained alcohol esters of fatty acids).
   e) Phosphatids.[2]
      1. Lecithins (glycerine esterified with 2 molecules of fatty acid and 1 molecule of phosphorylcholine).
      2. Cephalins (glycerine esterified with 2 molecules of fatty acid and 1 molecule of either phosphorylcolamine or phosphorylserine). Lipositol is a complicated cephalin-like substance with inositol as an additional component.
      3. Plasmalogens (glycerophosphorylcolamine [or choline] in a cyclic acetal linkage with 1 molecule of fatty aldehyde).
      4. Sphingomyelin (sphingosine esterified with 1 molecule of phosphorylcholine and in acylamide linkage with 1 molecule of fatty acid).
   f) Cerebrosides.[2] Sphingosine galactoside or glucoside in acylamide linkage with 1 molecule of fatty acid (examples: kerasin, phrenosin) are the simplest representatives of this group; there are also more complicated ones containing several units of various sugars and unidentified amino acid components. A special group of cerebrosides contains sulfuric acid (see the review of Blix[3] on sulfur-containing lipids).
D. Lipid peroxides.
E. Steroids.
   a) Cholesterol.

2. Page, J. H.: Chemistry of the brain (Springfield, Ill., and Baltimore, Md.: C. C. Thomas, 1937).
3. Blix, G.: Ztschr. f. physiol. Chem., **219**:82, 1933.

b) Cholesterol esters.
c) Steroid hormones.

F. Group of chemically unidentified lipid pigments, to be dealt with in greater detail in the section on pigments (lipofuscin, ceroid). These substances appear to be complicated polymerization products of unsaturated fatty acids. Their solubility in lipid solvents is variable; some of them will resist even embedding in paraffin.

It should be made clear that almost every group of substances mentioned includes a vast array of individual compounds. The theoretically possible number of glycerids and phosphatids, according to the laws of permutation, runs into the thousands because of the large variety of fatty acids which can combine. In addition, practically none of the individual groups occurs singly within morphological structures (droplets, mitochondria, etc.) but almost always as admixtures with other groups and in highly variable ratios.

The physical properties of pure substances may be markedly modified by admixtures. This is a very important point because some of the most widely used histochemical methods for the demonstration of lipids depend on the purely physical phenomenon of the solubility of certain dyes in them.

The two most important features of lipid substances affecting their stainability by dyes are their melting points and the presence or absence of hydrophilic groups (especially if the dye is applied in an aqueous solution). It is obvious that in the case of a solid, hydrophobic lipid a partition equilibrium between the lipid and the aqueous (or dilute alcoholic) phases can never be established, simply because the dye cannot penetrate the lipid particle to any appreciable depth. On the other hand, a lipid of oily consistency or one possessing hydrophilic groups may be stained through and through. All transitions between these two extremes are possible. It is easy to see that admixtures lowering the melting point or imparting a certain degree of hydrophilia will enhance the staining of the particles in bulk, whereas coating of other-

wise tingible substances by even a thin layer of water-repellent lipid may block the contact between the interior of the particle and the dye solution.

Owing to the similarity in the physical properties of the lipids as a group, sharp separation of the individual subgroups is a formidable task even in conventional preparative chemistry, and an impossibility in histochemistry. Some degree of selective removal or preservation of certain components can be accomplished, but the results are not even remotely quantitative. Another complicating factor is the protective action of proteins. For instance, kerasin is extractable from ground-up tissue by hot methyl alcohol or chloroform, but it is almost insoluble (especially after formalin fixation) while combined with protein within intact cells.[4]

This complexity of the situation makes the histochemical analysis of lipids very difficult and often illusory. Even the results of model experiments, which have been done in fairly large numbers[5, 6, 7, 8] since Altmann's original attempt in 1890, cannot be accepted at face value. The "pure" substances used were mostly biological concentrates the purity of which is highly questionable. If model experiments are to possess the force of proof, they must be performed with synthetic compounds the components of which (especially the fatty acid moiety) are exactly known. So far, such experiments have not been done on a comprehensive scale.

4. Morrison, R. W., and Hack, M. H.: Am. J. Path., **25**:597, 1949; Pearse, A. G. Everson: J. Clin. Path., **2**:81, 1949.

5. Altmann, R.: Die Elementarorganismen und ihre Beziehungen zu den Zellen (Leipzig: Veit & Co., 1890); Mulon, P.: Bibliog. anat., **3**:208, 1904; Camus, J., and Pagniez, P.: Compt. rend. Soc. de biol., **59**:701, 1905.

6. Smith, J. L., and Muir, W.: J. Path. & Bact., **13**:14, 1909.

7. Escher, H. H.: Corresp.-Blatt f. schweiz. Aerzte, **49**:1609, 1919; Kaufmann, C., and Lehmann, E.: Zentralbl. f. allg. Path. u. path. Anat., **37**:145, 1926; Kaufmann, C., and Lehmann, E.: Virchows Arch. f. path. Anat., **261**:623, 1926; Kaufmann, C., and Lehmann, E.: *ibid.*, **270**:360, 1928; Baker, J. R.: Quart. J. Micr. Sc., **88**:463, 1947.

8. Cain, A. J.: Quart. J. Micr. Sc., **89**:429, 1948.

Histochemical Methods for Lipids

Fixation.—In some cases (especially when true chemical reactions are employed) it may be preferable to use fresh, unfixed tissues, because fixation may modify the reactive groups. In other cases such modifying effects are used deliberately (see p. 101).

As a routine fixative, formalin is the best and simplest, especially when about 1 per cent $CaCl_2$ is added to render phospholipids insoluble (Baker).[9] Millot and Giberton[10] report that prolonged fixation in formalin will lead to a progressive decrease in the amount of total fat and also to an increase in the proportion of free fatty acid. The description of their experiments is not clear enough to permit a judgment as to the validity of their conclusions.

Of course, embedding either in celloidin or in paraffin will remove lipids more or less quantitatively unless they are made insoluble first (by special treatments, such as prolonged chromation). Embedding in carbowax (Blank,[11] Firminger[12]), on the other hand, appears to be a usable procedure. For most purposes it is simplest to use frozen sections. They should not be exposed to concentrations of alcohol higher than 70 per cent, and even this may cause the solution of the finest droplets.

The actual procedures of demonstration will be divided into two groups—physical and chemical.

Physical Methods

A) *Staining with oil-soluble dyes.*—There is a large number of oil-soluble dyes known, many of which are suitable for

9. Baker, J. R.: Quart. J. Micr. Sc., **87**:441, 1946.
10. Millot, J., and Giberton, A.: Compt. rend. Soc. de biol., **97**:1674, 1927.
11. Blank, H.: J. Invest. Dermat., **12**:95, 1949.
12. Firminger, H. J.: Stain Technol., **25**:121, 1950.

histological purposes (Sudan III and IV; Sudan black B;[13, 14] Oil red O;[15] Blue B.Z.L.;[13] Nile blue; etc.). With the exception of Sudan black B, which is an excellent stain for phosphatides, all the dyes mentioned stain triglycerides and fatty acids in a much darker shade than phospholipids. The latter may actually remain practically unstained. According to Gérard,[16] Sudan black B can be used to differentiate between petrolatum and animal lipids. The former will stain in a clear violet, the latter blue-black.

Oil dyes (with the exception of Nile blue) are usually made up in alcohol (50–70 per cent) or some similar solvent, such as ethylene or propylene glycol[17, 18] or pyridine.[19] Solutions in 70 per cent alcohol stain fast, but they may remove some of the lipid. Lower concentrations of alcohol are safer, but their solvent power for the dyes is lower, and optimal staining time is greatly prolonged. Solutions in glycols are reported to be safe and fast in action;[18] however, in view of the known solvent power of glycols (especially of propylene glycol) for many water-insoluble organic substances, the method should be re-examined critically. Sixty per cent isopropyl alcohol [20] and 50 per cent diacetin[21] have also been recommended as solvents.

The writer finds that one of the good solvents for oil dyes is 60 per cent triethylphosphate. It is entirely harmless to lipids, and the staining power of the solution is not much inferior to that of alcoholic solutions. In addition, the low volatility of triethylphosphate prevents the precipitation of

13. Lison, L.: Compt. rend. Soc. de biol., **115**:202, 1934.
14. Lison, L., and Dagnelie, J.: Bull. d'histol. appliq. à la physiol., **12**:85, 1935.
15. Lillie, R. D.: Stain Technol., **19**:55, 1944.
16. Gérard, P.: Bull. d'histol. appliq. à la physiol., **12**:92, 1935.
17. Hartman, T. L.: Stain Technol., **15**:23, 1940.
18. Chiffelle, T. L., and Putt, F. A.: Stain Technol., **26**:51, 1951.
19. Proescher, F.: Stain Technol., **2**:60, 1927.
20. Lillie, R. D., and Ashburn, L. L.: Arch. Path., **36**:432, 1943.
21. Gross, W.: Ztschr. f. wissensch. Mikr., **47**:64, 1930.

the dye caused by solvent evaporation, an occurrence often experienced with alcoholic solutions.

Method

Carry frozen sections in 50 per cent alcohol for a few minutes. Stain in a saturated and filtered solution of any of the dyes mentioned in 70 per cent alcohol or 60 per cent triethylphosphate for 5–20 minutes. Differentiate in 50 per cent alcohol for about 1 minute. Counterstain as desired. Mount in glycerin-gelatin or some similar medium.

Pure kerasin stains intensely with Sudan III, while the kerasin-protein complex of Gaucher cells does not. Franco and Wolman[22] find that boiling the sections at pH 4 for 30–60 seconds will break the complex and make the cells intensely sudanophilic.

Nile blue is different from the other dyes in several respects. First of all, it is water-soluble; second, it has two components: a blue oxazin and a red oxazone dye;[23–25] third, besides being an oil dye, it is also a regular basic dye, staining nuclei blue and mucin metachromatically pink.[26]

The value of Nile blue has been a subject of much debate ever since its introduction into histological technique by Lorrain Smith.[23] It appears, however, that the investigations of Cain[25] have settled the argument once and for all. Triglycerids, whether saturated or not, are colored red by the oxazone, provided that the temperature of staining is not below their melting points. All acidic lipids (including fatty acids and phospholipids) are stained blue because they bind the oxazin base (pink itself) in the form of blue salts. In mixtures, intermediate shades will be obtained.

22. Franco, S., and Wolman, M.: Schweiz. Ztschr. f. Path. u. Bakt., **10**:49, 1947.
23. Smith, J. L.: J. Path. & Bact., **12**:1, 1908.
24. Lison, L.: Bull. d'histol. appliq. à la physiol., **12**:279, 1935.
25. Cain, A. J.: Quart. J. Micr. Sc., **88**:383, 1947.
26. Baker, J. R.: Quart. J. Micr. Sc., **85**:1, 1944.

When using Nile blue, it should be remembered that it is not a specific lipid stain. While the Sudans stain lipids and nothing else, not all structures stained either red or blue by Nile blue are of a lipid nature.

B) *Fluorescence microscopy.*—Many lipid substances fluoresce in ultraviolet light (oxidation products of cholesterol and of various unsaturated fatty acids, vitamin A, ceroid, etc.), but only the fleeting green fluorescence of vitamin A is characteristic enough to be useful in histochemistry. For the technical details of the demonstration of vitamin A the reader is referred to the articles of Popper.[27]

A few attempts at the localization of vitamin A by chemical reactions, such as the Carr-Price test, appear to have been gross violations of the fundamental principles of histochemistry.[28] Tissues were dehydrated in alcohol and treated with a solution of antimony trichloride in chloroform. Of course, such a treatment would remove all lipids, vitamin A included, almost quantitatively. It is difficult to see how the positive reactions reported were obtained.

C) *Polarization microscopy.*—This used to be considered a valuable means for the distinction of doubly refractile cholesterol from other lipids. However, Lison[29] has shown that its results cannot be interpreted in a chemically meaningful way. In the case of lipids, birefringence appears to depend largely on factors other than chemical constitution (such as state of aggregation, supercooling, the nature of the mounting medium, etc.) even in the case of pure compounds; the behavior of mixtures is unpredictable. The sensitivity of the method is poor; lipid mixtures containing less than 5 per

27. Popper, H.: Proc. Soc. Exper. Biol. & Med., **43**:133, 1940; Popper, H.: Arch. Path., **31**:766, 1941; Greenberg, R., and Popper, H.: J. Cell. & Comp. Physiol., **18**:269, 1941.

28. Bourne, G.: Australian J. Exper. Biol., **13**:239, 1935; Joyet-Lavergne, P.: Protoplasma, **28**:131, 1937; Joyet-Lavergne, P.: Compt. rend. Soc. de biol., **126**:650, 1937; Jones, O. P.: J. Lab. & Clin. Med., **32**:700, 1947.

29. Lison, L.: Bull. d'histol. appliq. à la physiol., **10**:237, 1933.

cent cholesterol show no birefringence.[30] Even a positive result is not necessarily evidence of the presence of cholesterol.[31]

Chemical Methods

A) *For fatty acids and insoluble soaps (Ca).*—These substances can be demonstrated by Fischler's method.[32]

Method

Mordant frozen sections in a half-saturated solution of cupric acetate for 3–12 hours around 37° C.; rinse sections thoroughly in repeated changes of distilled water and stain them in a 0.5 per cent solution of hematoxylin in 50 per cent alcohol for 12–24 hours. Differentiate in Weigert's borax-ferricyanide mixture until the background (nuclei included) is decolorized. The cupric soaps are very resistant to decolorization and will remain almost black.

The reaction is not too specific; red cells, muscle, and calcareous deposits of any sort are also intensely stained. Two control sections can be used: one from which lipids are removed with warm methyl alcohol or chloroform (preferably with about 10 per cent acetic acid added to decompose the soaps) and another one treated with a citrate buffer of pH 4.5–5 to remove other calcium deposits.

The use of Nile blue has been mentioned. Fauré-Fremiet[33] has reported that unsaturated fatty acids stain metachromatically with methyl green and light green; his findings could not be confirmed.

B) *For cholesterol and its esters.*—

a) Schultz's method[34] is the histochemical application of

30. Okey, R.: J. Biol. Chem., **156**:179, 1944.

31. Yoffey, J. M., and Baxter, J. S.: J. Anat., **81**:335, 1947.

32. Fischler, F.: Zentralbl. f. allg. Path. u. path. Anat., **15**:913, 1904.

33. Fauré-Fremiet, Mayer A., and Schaeffer, G.: Arch. d'anat. micr., **12**:19, 1910.

34. Schultz, A.: Zentralbl. f. allg. Path. u. path. Anat., **35**:314, 1924–25; Schultz, A., and Löhr, G.: Zentralbl. f. allg. Path. u. path. Anat., **36**:529, 1925.

the Liebermann-Burchardt test, which is positive with all unsaturated sterols,[35] whether esterified or not. For all practical purposes, it may be considered a specific test for cholesterol and its esters.

Method

Mordant frozen sections in 2 per cent ferric alum for 24 hours. This step is essential, although its chemical background is not well understood. Rinse sections in distilled water. Mount them on slides, blot them dry. Place a few drops of a mixture of equal parts of glacial acetic acid and concentrated sulfuric acid (*caution:* cool test tube while mixing the acids!) on the section and cover it with a cover slip. A change of colors from purple-red through dark blue to blue-green will take place within about 1 minute. Only the last shade mentioned is diagnostic for cholesterol.

b) The digitonin reaction[36] is specific for unesterified 3-cis-OH sterols, such as cholesterol, the vitamin D compounds, isoandrosterone, etc.; 3-trans-OH compounds (androsterone, bile acids) do not react.

Method

Immerse frozen sections for a few hours in a 0.5 per cent solution of digitonin in 50 per cent alcohol. Wash in 50 per cent alcohol and in water. Mount in glycerin-jelly. Under the polarizing microscope typical groups of fine needle-shaped crystals are seen; they are birefringent.

C) *For carotenoids.*—One of the carotenoids, vitamin A, has been mentioned under "Fluorescence microscopy." Carotenoid pigments will be considered in the chapter on "Pigments."

35. Sobotka, H.: The chemistry of the sterids (Baltimore: Williams & Wilkins, 1938), p. 158.

36. Brunswik, H.: Ztschr. f. wissensch. Mikr., **39**:316, 1922; Leulier, A., and Noel, R.: Bull. d'histol. appliq. à la physiol., **3**:316, 1926; Leulier, A., and Revol, L.: Bull. d'histol. appliq. à la physiol., **7**:241, 1930; Lison, L.: Histochimie animale (Paris: Gauthier-Villars, 1936).

D) *For unsaturated fatty acids.*—Unsaturated fats are slowly oxidized even when injected into the tissues; more readily on exposure to atmospheric oxygen; and very rapidly in the presence of various oxidants and catalysts. The products of oxidation may include a large variety of compounds[37–40] such as peroxides, epoxides, aldehydes, possibly ketones, hydroxyacids, various fragments and polymers. Some of the latter may be colored and/or insoluble in lipid solvents. The insoluble fraction may exhibit surprising staining reactions, such as acid-fastness and affinity to orcein and resorcinol-fuchsin.[41] The chemistry of the oxidative process is poorly understood; the factors which influence the rate of oxidation and the nature of the end-products are obscure.

Methods for unsaturated fatty acids are relatively specific for phospholipids and cholesterol esters because of the marked unsaturation of these compounds.

a) *Osmium tetroxide* (*osmic acid*).—This substance has been employed for the demonstration of triolein and oleic acid since 1895.[42] While oxidizing the ethylenic double bonds, it is reduced to a black substance (probably a mixture of lower oxides). It is of some value only if used according to the specifications of Cain[1] (well-washed frozen sections fixed briefly in formalin-$CaCl_2$ and treated with a solution of osmic acid for about 1 hour; avoidance of dichromate and alcohol). Even so, phospholipids containing oleic acid may react feebly or not at all.

b) *Dichromate methods.*—Potassium dichromate has been

37. Cummings, M. J., and Mattill, H. A.: J. Nutrition, **3**:421, 1930–31.

38. Bloor, W. R.: Biochemistry of the fatty acids (New York: Reinhold Pub. Co., 1943).

39. Markley, K. S.: Fatty acids (New York and London: Interscience Publishers, 1947); Hilditch, T. P.: The chemical constitution of natural fats (New York: John Wiley & Sons, 1947).

40. Ralston, A. W.: Fatty acids and their derivatives (New York: John Wiley & Sons, 1948).

41. Hass, G. M.: Arch. Path., **27**:15, 1939; Hass, G. M.: *ibid.*, **28**:177, 1939.

42. Starke, J.: Arch. f. Physiol., p. 70, 1895.

used in microscopic technique as a "hardener," especially of brain, for about one hundred years. Its effect on lipids consists in their oxidation to a miscellany of compounds, some of which are so insoluble as to be demonstrable by Sudan dyes even in paraffin sections (Ciaccio).[43] Dichromate itself is reduced at the sites of oxidation to an insoluble compound (Cr_2O_3?) which can combine with hematoxylin to form a blue-black lake, quite resistant to differentiation.

The rate at which unsaturated lipids chromate varies considerably. It appears that the presence of hydrophilic groups enhances chromation; e.g., phospholipids chromate readily,[44] and triolein chromates much faster if it contains a small amount of cholesterol than in a pure state.[45] This is not surprising, since potassium dichromate is lipid-insoluble.

The optimal conditions for the chromation of phospholipids have been studied carefully by Baker.[9] As a result of his studies, he succeeded in devising a sensitive and specific modification of Lorrain Smith's method.[6] The claims of specificity have been confirmed by Cain.[46]

Method

1. Fix in formalin containing 1 per cent $CaCl_2$.
2. Transfer pieces directly to a 5 per cent solution of potassium dichromate containing 1 per cent of $CaCl_2$ and keep them in it for 18 hours.
3. Continue mordanting in the same solution for 24 hours at 60° C.
4. Cut frozen sections, preferably after gelatin-embedding.
5. Mordant sections in the dichromate-calcium solution for 1 hour at 60° C.
6. Wash sections in water and stain them in the following, freshly prepared, solution for 5 hours at 37° C.:

43. Ciaccio, C.: Zentralbl. f. allg. Path. u. path. Anat., **20**:385, 1904.
44. Ciaccio, C.: Compt. rend. A. anat., **25**:87, 1930.
45. Dietrich, A.: Verh. d. deutsch. path. Gesellsch., **14**:263, 1910.
46. Cain, A. J.: Quart. J. Micr. Sc., **88**:467, 1947.

Dissolve 50 mg. of hematoxylin in 48 ml. of distilled water, add 1 ml. of a 1 per cent solution of potassium iodate, and heat it to a boil. Cool, add 1 ml. of acetic acid.

7. Rinse sections, differentiate in a 0.25 per cent solution each of borax and of potassium ferricyanide for 18 hours at 37° C.

8. Wash and mount in glycerin-jelly.

Lecithin, cephalin, and sphingomyelin stain in an intense blue-black shade. Only this shade is diagnostic; other unsaturated lipids may stain in shades of brown and gray. A few nonlipid substances, such as mucin and hemoglobin, will stain in the same shade as the phospholipids. As a control, Baker suggests the extraction of an adjacent tissue block, fixed in Bouin's solution, with pyridine at 60° C., and running it through the same schedule. Since this treatment removes all lipid, whatever is stained is nonlipid. The difference between the two blocks is due to phosopholipid only.

Valade[47] recommends a different scheme of extraction and chromation; its specificity for phopholipids has not been tested.

Chromated tissues can also be embedded in paraffin and stained with Sudan dyes, preferably Sudan black B. This method does not differentiate clearly between phospholipids and other unsaturated fats, although the former (since they chromate faster and are rendered insoluble more readily) will stain more intensely. Quantitative preservation of any type of lipid cannot be expected.

c) Alsterberg[48] proposes the demonstration of phospholipids by a different principle. The reagent consists of cyanogen iodide and silver nitrate (or chlorate). According to the author, choline and colamine decompose cyanogen iodide and cause the precipitation of a mixture of silver cyanide and iodide which can be visualized in a second step. This method requires further investigation; neither the soundness

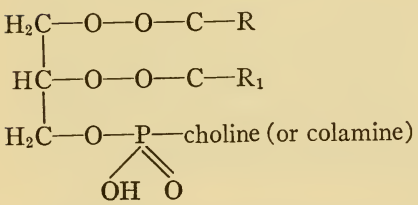
47. Valade, P.: Bull. Acad. vét. France, **22**:77, 1949.

48. Alsterberg, G.: Ztschr. f. Zellforsch. u. mikr. Anat., **31**:364, 1940–41.

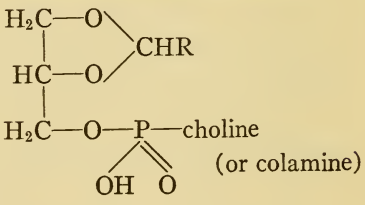
of the chemical background nor the specificity of the results has been tested so far.

E) *Lipid aldehydes.*—In 1924 Feulgen and Voit,[49] while studying the nucleal reaction in $HgCl_2$-fixed tissues, noticed a widespread staining of elastic membranes and various cytoplasmic structures by Schiff's reagent, even without preceding acid hydrolysis. Staining could be prevented by pretreatment with phenylhydrazine. Obviously, the reaction was due to some hitherto undescribed aldehyde to which they gave the name of "plasmal," and the unknown compound from which it is set free by $HgCl_2$ they called "plasmalogen." Further studies by Feulgen and his group[50] gradually succeeded in unraveling the nature of these mysterious substances. Plasmalogen turned out to be a new type of compound, an acetalphosphatide. Acetalphosphatides resemble lecithin or cephalin except for the fact that they contain only one molecule of fatty aldehyde per molecule of glycerol, and the linkage between the fatty aldehyde and glycerol is of the cyclic acetal type.

alpha-Lecithin



alpha-Acetalphosphatide



(R and R_1, fatty acid radicals)

49. Feulgen, R., and Voit, K.: Arch. f. d. ges. Physiol., **206**:389, 1924.

50. Feulgen, R., and Imhäuser, K.: Biochem. Ztschr., **181**:30, 1927; Imhäuser, K.: Biochem. Ztschr., **186**:360, 1927; Voss, H.: Ztschr. f. mikr.-anat. Forsch., **10**:583, 1927; Behrens, M.: Ztschr. f. physiol. Chem., **191**:183, 1930; Feulgen, R., and Behrens, M.: Ztschr. f. physiol. Chem., **256**:15, 1938; Feulgen, R., and Bersin, T.: Ztschr. f. physiol. Chem., **260**:217, 1939; Voss, H.: Ztschr. f. Zellforsch. u. mikr. Anat., **31**:43, 1940–41; Bersin, T., Moldtman, H. G., Nafziger, H., Marchand, B., and Leopold, W.: Ztschr. f. physiol. Chem., **269**:241, 1941.

The acetal linkage is slowly hydrolyzed by acids and oxidizing agents but promptly by heavy-metal salts such as $HgCl_2$, with liberation of the corresponding aldehyde. Actually, plasmal has been shown to be a mixture of several long-chained aldehydes, mainly stearic and palmitic, with the admixture of unidentified unsaturated ones.

Plasmal will react with all aldehyde reagents such as Schiff's, phenylhydrazine, semicarbazide, naphthoic hydrazide, etc. Highly colored reaction products (with 2,4-dinitrophenylhydrazine, Schiff's reagent, or 2-hydroxy-3-naphthoic hydrazide followed by azo-coupling[51–53]) can be used for its histochemical identification.

It should be mentioned, however, that plasmal is not the only lipid aldehyde in the tissues.[54–56] Substances of aldehydic nature are formed in large amounts by the oxidation of unsaturated fatty acids.[38–40] The presence of such nonplasmal aldehydes can be demonstrated (some of them at specific sites; Chu)[57] even in completely fresh tissues. Their amount increases and their distribution becomes more widespread on storage in either formalin or water. The effect is probably due to atmospheric oxygen, since it is especially marked in sections kept in a shallow layer of fluid. Intense reactions are obtained at all sites where cholesterol or phospholipids are present. This is not surprising, in view of the fact that the fatty acids of cholesterol esters[58] and of phopholipids[59] show

51. Camber, B.: Nature, **163**:285, 1949.

52. Seligman, A. M., and Ashbel, R.: Bull. New England M. Center, **11**:85, 1949.

53. Seligman, A. M., Friedman, O. M., and Herz, J. E.: Endocrinology, **44**:584, 1949.

54. Cain, A. J.: Quart. J. Micr. Sc., **90**:75, 1949.

55. Danielli, J. F.: Quart. J. Micr. Sc., **90**:67, 1949.

56. Hayes, E. R.: Stain Technol., **24**:19, 1949.

57. Chu, C. H. U.: J. Nat. Cancer Inst., **10**:1344, 1950; Chu, C. H. U.: Anat. Rec., **108**:723, 1950.

58. Kelsey, F. E., and Longenecker, H. E.: J. Biol. Chem., **139**:727, 1941.

59. Page, I. H., and Rudy, H.: Ztschr. f. Physiol. Chem., **205**:115, 1932.

a high degree of unsaturation. On prolonged storage, even depot fat will develop more or less intense aldehyde reactions. $HgCl_2$ has little effect on the formation of aldehyde from unsaturated fatty acids. Periodic acid, on the other hand, produces large amounts of aldehyde.[60] Plasmalogen as a source of aldehydes is probably much less important quantitatively than unsaturated fatty acids.

The question is whether there may be any nonaldehydic substances in the tissues which could give positive reactions with the reagents mentioned.

The first group of compounds to be considered are the ketones. Some of them may result form the oxidation of unsaturated fatty acids (especially by the rearrangement of epoxides[61]), but there is no positive proof for their existence in animal tissues. On the other hand, ketosteroids are known to be present in a number of tissues. They give no color with Schiff's reagent,[62, 63] which is fairly specific for aldehydes; but there can be no doubt that they would give good positive reactions with most other carbonyl reagents. Actually, the reaction obtained with phenylhydrazine[64] or with 2-hydroxy-3-naphthoic hydrazide[51] has been assumed to be indicative of the presence of ketosteroids. However, the evidence adduced is of only a circumstantial nature. Dempsey[65] says that, while not one of the physical and chemical features (carbonyl reactions, Schultz test, birefringence, fluorescence, solubility in

60. Wolman, H.: Proc. Soc. Exper. Biol. & Med., **75**:583, 1950.

61. Fourneau, E., and Tiffeneau, M.: Compt. rend. Acad. sc., **144**:662, 1905.

62. Albert, S., and Leblond, C. P.: Endocrinology, **39**:386, 1946.

63. Oster, K. A., and Oster, J. G.: J. Pharmacol. & Exper. Therap., **87**:306, 1946.

64. Bennett, H. S.: Proc. Soc. Exper. Biol. & Med., **42**:786, 1939; Bennett, H. S.: Am. J. Anat., **67**:151, 1940; Dempsey, E. W., and Bassett, D. L.: Endocrinology, **33**:384, 1943; Deane, H. W., and McKibbin, J. M.: Endocrinology, **38**:385, 1946; Deane, H. W., and Greep, R. O.: Am. J. Anat., **79**:117, 1946; Greep, R. O., and Deane, H. W.: Anat. Rec., **97**:416, 1947; Greep, R. O., and Deane, H. W.: Ann. New York Acad. Sc., **50**:596, 1949; Applegarth, A.: Endocrinology, **44**:197, 1949.

65. Dempsey, E. W.: Recent Prog. Hormone Research, **3**:127, 1948.

acetone, sudanophilia) of the lipid present at sites of ketosteroid production is really specific for ketosteroids, no other known type of substance displays the entire battery of reactions. Chemical considerations are decidedly against this assumption. In fresh tissues such reactions are entirely negative; on mere standing or after treatment with mild oxidants they become increasingly positive—an indication that whatever gives the reaction must be the result of an oxidative process. Ketosteroids would be expected to react directly, even in fresh tissues. The distribution pattern of carbonyl compounds as seen with the relatively aldehyde-specific Schiff's reagent is invariably either identical with, or extremely similar to, that obtained by the use of "ketone reagents," both in the endocrine organs and elsewhere[66] (brain, atherosclerotic plaques, necrotic tumors, etc.). The minor discrepancies occasionally observed are theoretically readily explainable by differences in the lipid solubility of the reagents and possibly by differences in reactivity of the several aldehydic substances present (steric factors?). Similar differences in reactivity have been reported in the cases of such relatively homogeneous groups of substances as desoxyribosenucleic acids[67] and mucopolysaccharides,[68] even though the reactive groups within each class are the same.

In a series of recent papers[69] Seligman and his group reported their studies on the carbonyl groups of formalin-fixed nervous tissue, adrenals, and testes, demonstrated by the 2-hydroxy-3-naphthoic acid hydrazide-Blue B Salt method. They came to the conclusion that the reacting carbonyl groups, "unmasked" by formalin, are of a ketonic rather than

66. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **51**:133, 1942.

67. Lessler, M. A., and Kopac, M. J.: Anat. Rec., **108**:578, 1950.

68. Monné, L., and Slautterback, D. B.: Exper. Cell Research, **1**:477, 1950.

69. Ashbel, R., and Seligman, A. M.: Endocrinology, **44**:565, 1949; Seligman, A. M., and Ashbel, R.: Cancer, **4**:579, 1951; Seligman, A. M., and Ashbel, R.: Endocrinology, **49**:110, 1951; Ashbel, R., Cohen, R. B., and Seligman, A. M.: Endocrinology, **49**:265, 1951; Rabinovici, N.: Endocrinology, **49**:579, 1951.

of an aldehydic character. The main points of their arguments are as follows:

1. It can be shown that the reaction is not due to plasmal, since treatment of unfixed tissue by mercuric chloride causes only a weak reaction.

2. Phenylhydrazones of aldehydes react with diazonium salts to form intensely colored formazans, while ketonic phenylhydrazones, in the absence of replaceable hydrogen, cannot couple with diazonium salts. This formazan reaction is negative with nerve tissue, adrenal lipid, etc.

3. Aniline and sulfanilic acid, according to Oster and Mulinos[70] and Boscott and Mandl,[71] selectively block aldehydes. The carbonyl groups in question are not blocked under the conditions specified.

4. The Angeli-Rimini test[72] for aldehydes is negative with the tissues.

The problem of lipid aldehydes versus ketones has been subjected to an experimental study by Gomori.[73] His findings are as follows:

1. It can be shown in model experiments that fluorescence is a property of auto-oxidation products rather than of ketosteroids.

2. Positive carbonyl reactions can be obtained after formalin-free fixatives, such as a saturated solution of picric acid in 50 per cent alcohol. Therefore, "unmasking" by formalin is a superfluous assumption.

3. Seligman's criteria for the nonaldehydic nature of lipid carbonyl groups are not valid. Formazans do not form from all aldehydes; for instance, periodate-treated glycogen is entirely negative. The results of blocking experiments permit

70. Oster, K. A., and Mulinos, M. G.: J. Pharmacol. & Exper. Therap., **80**:132, 1944.

71. Boscott, R. J., and Mandl, A. M.: J. Endocrinol., **6**:132, 1949.

72. Angeli, A., and Angelico, F.: Gazz. chim. ital., **34-I**:50, 1904; Rimini, E.: Atti reale Accad. Lincei, ser. 5, cl. di sc. fis., mat. e nat., **17-II**:360, 1908.

73. Gomori, G.: J. Lab. & Clin. Med., **39**:649, 1952.

no conclusions whatsoever as to the aldehydic or ketonic nature of carbonyl groups. The Angeli-Rimini reaction is known to be negative with a number of aldehydes even in test-tube experiments;[74] it is invariably negative histochemically with unquestionably aldehydic substances.

4. Estrone, testosterone, and desoxycorticosterone give negative reactions with Seligman and Ashbel's reagent under the conditions of the histochemical test.

5. The carbonyl reactions in the tissues are so intense that they must be due to a substance which constitutes a substantial percentage of the lipid material.

On the basis of these findings, Gomori comes to the conclusion that the "battery of reactions" is due not to ketosteroids but to auto-oxidation products of unsaturated fatty acid esters of cholesterol.

In summary, it may be said that, for the time being, we do not possess a single reaction capable of identifying ketones, let alone ketosteroids, in the midst of a large bulk of lipid aldehydes. This view is shared by the vast majority of workers who have studied the problem critically (Gomori;[66] Aboim;[75] Albert and Leblond;[62] Claesson and Hillarp;[76] Boscott, Mandl, Danielli, and Shoppee;[77] Boscott and Mandl;[71] and Sayers[78]). The invariable association of ketosteroids with aldehydes (but not vice versa) simply means that the steroid hormones are handled metabolically, at least up to a certain point, very much like cholesterol.

In addition to aldehydes and ketones, a number of other compounds have been asserted to give a positive test with Schiff's reagent. According to Lison,[79] ethylenic double bonds

74. Angeli, A., and Angelico, F.: Gazz. chim. ital., **33-II**:245, 1903; Angeli, A., and Marchetti, G.: Atti reale Accad. Lincei, ser. 5, cl. di sc. fis., mat. e nat., **17-II**:360, 1908.

75. Aboim, A. Nunes: Bull. Soc. port. sc. nat., **14**:119, 1943.

76. Claesson, L., and Hillarp, N. A.: Acta anat., **3**:109, 1947.

77. Boscott, R. J., Mandl, A. M., Danielli, J. F., and Shoppee, C. W.: Nature, **162**:572, 1948.

78. Sayers, G.: Physiol. Rev., **30**:241, 1950.

79. Lison, L.: Bull. d'histol. appliq. à la physiol., **9**:177, 1932.

(such as in oleic and cinnamic acids) may react. However, this is denied by Verne[80] and Oster and Oster,[63] who find that a positive reaction is always due to contamination by products of oxidation.

Gérard[81] and Verne[82] made the observation that the same lipid structures which stain positively with Schiff's reagent also show strong oxidative properties, e.g., they recolorize leuco-bases and stain intensely blue with the nadi-reagent, although the intensity of the aldehyde and oxidative reactions need not run parallel. This interesting observation may be explained by the invariable compresence of aldehydes and peroxides; furthermore, there is a possibility that part of the color obtained in the Schiff reaction is due to a pseudo-reaction: the oxidative recolorization of the reagent. However, differentiation should be easy; the true aldehyde color is extremely resistant to acids, whereas regenerated fuchsin is readily decolorized by them.

Histochemical methods for lipid aldehydes

1) *For pre-formed aldehydes.*—The best method is to use fresh, unfixed frozen sections. However, a few hours' fixation in formalin, followed by thorough washing in repeated changes of distilled water, is permissible, although the staining of some structures (e.g., myelin sheaths) may be greatly weakened.[83] Freshly boiled distilled water should be used to make up the formalin solution and to rinse the sections.

Stain in Schiff's reagent (straight or diluted with an equal volume of distilled water) for about 30–60 minutes. Rinse sections in a 1–3 per cent solution of Na bisulfite for a few minutes. Rinse in repeated changes of tap water. Counterstain with hematoxylin. Mount either in glycerin-jelly or, after dehydration, in balsam. The fatty aldehyde–Schiff compound is lipid-insoluble.

80. Verne, J.: Ann. de physiol., **5**:245, 1929.

81. Gérard, P.: Bull. d'histol. appliq. à la physiol., **12**:274, 1935.

82. Verne, J.: Compt. rend. Soc. de biol., **133**:75, 1940; Verne, J.: Bull. d'histol. appliq. à la physiol., **13**:433, 1936.

83. Verne, J.: Bull. d'histol. appliq. à la physiol., **14**:269, 1937.

2) *For acetalphosphatides.*[55]—Use unfixed or formalin-fixed tissues. Block pre-formed aldehydes by treating the sections with a 2 per cent solution of either phenylhydrazine or hydroxylamine-HCl in an acetate buffer of pH 4.5–5.5 for 6–12 hours at room temperature. Rinse thoroughly in distilled water. Treat sections with a 2 per cent solution of $HgCl_2$ for 15 minutes. Rinse in distilled water. Stain, etc., as under 1, above.

Danielli[55] finds that hydrolysis in 0.1 N HCl for 15 minutes at room temperature is preferable to treatment with $HgCl_2$ because the latter may have oxidative side effects.

3) *For aldehydes secondary to the oxidation of unsaturated fatty acids.*[55]—Treat sections with $HgCl_2$ or 0.1 N HCl; block aldehydes with one of the reagents mentioned under 2, above; wash and expose them to air for several days (for instance, in a flat dish containing a shallow layer of water). Stain, etc., as under 1, above.

It is always advisable to extract a control section with several changes of alcohol-ether or acetone and to carry it through the entire procedure. The fat-free blank will help to rule out reactions given by nonlipid substances.

C. PROTEINS, AMINO ACIDS, AND PRODUCTS OF PROTEIN METABOLISM

The demonstration of the presence of protein substances as such is of relatively minor importance in histochemistry because proteins are ubiquitous components of all tissues. Occasionally, however, it may be necessary to ascertain the protein or nonprotein nature of certain structures, such as granulations. The identification of the individual amino acids is of much more interest but possible only in a few special instances.

Proteins

The methods for the demonstration of proteins can be divided into two groups: precipitation and digestion tests.

1. The precipitation tests are based on the fact that many proteins retain their affinity for protein precipitants even

though they have been precipitated previously by histological fixatives.

a) *The ferrocyanide reaction of Hartig-Zacharias.*[1]

Method

Treat the section for 10 minutes with an acidified solution of potassium ferrocyanide (1–5 per cent in dilute hydrochloric acid; concentrations not important); wash thoroughly and flood with a dilute solution of ferric chloride. Proteins stained blue. Ferric compounds (hemosiderin) will be blue before the application of ferric chloride.

b) *The tannin-ferric method of Salazar.*[2]—

Method

Treat the section for 15–20 minutes with an acidified solution of tannin (5–10 per cent tannin in 5–10 per cent acetic acid; concentrations not important); wash thoroughly and flood with a dilute solution of ferric chloride. Some protein structures, secretion granules, etc., stain gray-black.

2. The digestion tests, once widely used, yield very little information of a chemical nature, although they do differentiate between various protein substances. Pepsin digests collagen and reticulum; trypsin, elastic fibers. For details the interested reader is referred to the review of the topic by Spalteholz and others.[3] This topic should be reinvestigated with the aid of purified proteolytic enzymes which have become available recently.

Amino Acid Components of Proteins

Of the reactions described for the histochemical identification of amino acids, only two are reagents for amino acids proper (ninhydrin and alloxan). The others are specific

1. Zacharias, E.: Bot. Ztschr., **41**:208, 1883.
2. Salazar, A. L.: Compt. rend. Soc. de biol., **83**:1655, 1920.
3. Verdauung, künstliche. In Enzykl. d. mikr. Technik (3d ed.; Berlin and Vienna: Urban & Schwarzenberg, 1927), **3**:2220.

either for the aromatic nucleus or for phenolic functions or for the guanidine grouping.

The shades given by any of the reagents to be mentioned cannot be compared for intensity and sharpness with those of the better color reactions in histochemistry. Some of the reagents will react only with unfixed (or incompletely fixed) proteins and, even so, give shades so pale and indistinct as to make the test virtually worthless. With some of the other tests, the interpretation of the results is vague. With the exception of the Millon and Sakaguchi reactions, which do give valuable information, the methods in this group will be mentioned for the record rather than because of their usefulness.

1. Millon's reaction[4] is one of the oldest tests for protein substances. Actually, the reagent is specific for phenols and, in the case of amino acids, for tyrosine.

Of the several modifications published,[5] Pollister's appears to be the most reliable.

Method

Any good fixation is suitable. Incubate sections at 30°–37° C. in a solution containing 5 per cent mercuric acetate and 15 per cent trichloroacetic acid. After 5–10 minutes add about one-tenth volume of a 1 per cent solution of sodium nitrite and incubate sections for another 25 minutes. Rinse sections directly in 70 per cent alcohol, dehydrate, and mount. Tyrosine-containing proteins are stained in a shade of pink to brick-red. The shade is rather transparent, and it is advisable, for better visibility, to use sections not thinner than 10 μ.

2. The Sakaguchi test[6] is specific for derivatives of guanidine in which at least one hydrogen in each of the amino

4. Millon, E.: Compt. rend. Acad. sc., **28**:40, 1849.

5. Bensley, R. R., and Gersh, I.: Anat. Rec., **57**:217, 1933; Serra, J. A., and Queiroz Lopes, A.: Port. acta biol., **1**:51, 1945; Pollister, A. W.: Rev. d'hématol., **5**:527, 1950.

6. Sakaguchi, S.: J. Biochem. (Japan), **5**:25, 1925.

groups is unsubstituted. Arginine is the only compound found in tissues which will give a positive reaction.

Of the several modifications published,[7] Baker's is relatively the simplest and most reliable.

Method

Use celloidin sections or paraffin sections protected with collodion. The thickness of the sections should be at least 10 μ.

Mix rapidly 2 ml. of 1 per cent NaOH, 2 drops of a 1 per cent solution of α-napthol in 70 per cent alcohol, 4 drops of a 1 per cent solution of Na hypochlorite (a dilution of Clorox or of some similar household bleaching agent with 7–10 parts of water). Pour mixture on the slide immediately and leave it on for 15 minutes. Drain fluid off the slide, blot it, and immerse it in a mixture of 3 parts of pyridine and 1 part of chloroform. Mount in the same medium. Arginine-containing proteins stain in an orange-red shade. The full intensity of the color is permanent for a few hours only. Clearing in xylene and mounting in balsam is permissible, but fading of the color will be even faster.

3. The xanthoprotein reaction[8] is specific for aromatic rings, which are nitrated by cold fuming nitric acid to yield yellow dyes.

4. Diazonium salts will couple with phenols and heterocyclic rings[9] (tyrosine, histidine, proline, etc.) to yield yellowish or brownish azo dyes of low color intensity.

5. Syrupy phosphoric acid, especially with a trace of some

7. Serra, J. A.: Port. acta biol., **1**:1, 1944; Serra, J. A.: Naturwiss., **32**:46, 1944; Thomas, L. E.: J. Cell. & Comp. Physiol., **28**:145, 1946; Baker, J. R.: Quart. J. Micr. Sc., **88**:115, 1947; Thomas, L. E.: Stain Technol., **25**:143, 1950; Warren, T. N., and McManus, J. F. A.: J. Nat. Cancer Inst., **12**:223, 1951.

8. Raspail, F. V.: Nouveau système de chimie organique (3d ed.; Brussels: 1840), **1**:161.

9. Pauly, H.: Ztschr. f. physiol. Chem., **42**:508, 1904; Brunswik, H.: Ztschr. f. physiol. Chem., **127**:268, 1923; Berg, W.: Arch. f. d. ges. Physiol., **199**:656, 1923.

aldehyde (vanillin, furfural, etc.) added, gives with tryptophane at 60°–65° C. a fairly intense but unstable purple-red color (Romieu's reaction[10]).

6. Ehrlich's *p*-dimethylaminobenzaldehyde reagent[11, 12] condenses with a number of phenols, amines, pyrrole and indole derivatives[13] to give reddish and bluish dyes of unknown constitution.

7. α-amino acids give, when boiled with a 0.2–0.5 per cent solution of ninhydrin (triketohydrindene hydrate), a blue coloration.[14] This method works only with tissues not too thoroughly fixed and unembedded. The color of the reaction has a great tendency to diffuse.

8. Alloxan in aqueous or alcoholic solutions gives a red color with α-amino acids.[15] The shade is so pale as to make the reaction worthless for histochemical purposes.

9. Voss[16] recommends the use of *o*-diacetylbenzene (10 per cent in 70 per cent alcohol) as a reagent for amino acids and proteins (bluish-red color).

Reactions 6–9, being specific for NH_2 groups, will be negative after formalin fixation.[11, 17]

An entirely new avenue for the histochemical investigation of protein substances is proposed by Danielli.[18] He recommends the application of a number of important condensation reactions of organic chemistry so modified as to yield

10. Romieu, M.: Compt. rend. Acad. sc., **180**:875, 1925; Blanchetière, A.: Compt. rend. Acad. sc., **180**:2072, 1925; Blanchetière, A., and Romieu, M.: Compt. rend. Soc. de biol., **107**:1127, 1931.

11. Ehrlich, P.: Med. Wchnschr., p. 151, 1901.

12. Lison, L.: Histochimie animale (Paris, 1936), p. 160.

13. Rohde, E.: Ztschr. f. physiol. Chem., **44**:161, 1905; Fleig, M. C.: Bull. soc. chim. France, 4th ser., **3**:1038, 1908.

14. Ruhemann, S.: J. Chem. Soc., Tr., **97**:2025, 1910; Berg, W.: Klin. Wchnschr., **2**:1757, 1923.

15. Krasser, F.: Monatschr. f. Chem., **7**:673, 1886; Hurtley, W. H., and Wootton, W. O.: J. Chem. Soc., Tr., **99**:288, 1911; Giroud, A.: Protoplasma, **7**:72, 1929.

16. Voss, H.: Ztschr. f. mikr.-anat. Forsch., **49**:51, 1940.

17. Dulière, W. L.: Biochem. J., **30**:770, 1936.

18. Danielli, J. F.: Cold Spring Harbor Symp. Quant. Biol., **14**:32, 1950.

colored end-products. Only two examples will be quoted to give an idea of the nature of the new tests:

1. Amino groups of the protein are condensed with aminobenzaldehyde; the aromatic amino group of the resulting Schiff's base can be diazotized and coupled with napthol.

2. Phenolic groups of the protein are condensed with dinitrochlorobenzene;[19] the nitro groups are reduced to amino groups and the latter coupled with a diazonium salt.

In both cases the final product will be an intensely colored azo dye. Theoretically, this approach is sound and can be expected to give excellent results.

Antigens

The microscopic localization of antigens in tissue sections is one of the newest and most promising fields of histochemistry.

The histochemical localization of antigens depends on the fact that it is possible to introduce various chemical groups into proteins without greatly changing their immunological properties.[20–23] If an antibody is coupled, e.g., with tetrazotized benzidine-R-salt conjugate, a red azoprotein is obtained which will be selectively adsorbed to the original antigen. This method has been used for *in vitro* agglutination studies.[24] Unfortunately, the shade of the azoprotein cannot be made dark enough to produce a satisfactory color contrast in combination with the appropriate antigen in tissue sections.

In 1941 Coons and associates developed a much more sensitive method by coupling antibodies with anthryl isocy-

19. Bost, R. W., and Nicholson, F.: J. Am. Chem. Soc., **57**:2368, 1935.
20. Landsteiner, K., and Lampl, H.: Biochem. Ztschr., **86**:343, 1918.
21. Heidelberger, M., and Kendall, F. E.: Science, **72**:252, 1930.
22. Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M.: J. Exper. Med., **58**:137, 1933.
23. Reiner, L.: Science, **72**:483, 1930; Heidelberger, M., and Kendall, F. E.: J. Exper. Med., **59**:579, 1934.
24. Marrack, J.: Nature, **133**:292, 1934.

anate[25] and later with fluorescein isocyanate.[26] In this way intensely fluorescent carbamido-proteins can be produced, which, if adsorbed on morphological structures, are easily visible under the microscope.

The method is rather complicated. Fluorescein isocyanate itself is not available on the market but must be synthesized by a laborious procedure; the conjugated antibody must be carefully purified to eliminate its nonspecific components. However, the method has already produced most valuable results in the localization of several antigens[27] (among others, ACTH)[28] in the tissues.

Urea

Urea is precipitated by a solution of xanthydrol in acetic acid as dixanthydryl urea, which forms beautiful rosettes of small needle-shaped crystals. The crystals are insoluble in water and in most organic solvents.

This reaction is the only really specific test for urea.[29] Unfortunately, its usefulness in histochemistry is limited because (1) the solvent severely damages histological structure and (2) the reaction is relatively slow, permitting considerable dilution and displacement of urea before precipitation occurs. For this reason the sensitivity of the reaction is low, and the localization is only approximate. The failure of the test to

25. Coons, A. H., Creech, H. J., and Jones, R. N.: Proc. Soc. Exper. Biol. & Med., **47**:200, 1941.

26. Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E.: J. Immunol., **45**:159, 1942.

27. Coons, A. H., and Kaplan, M. H.: J. Exper. Med., **91**:1, 1950; Kaplan, M. H., and Coons, A. H.: J. Exper. Med., **91**:15, 1950; Coons, A. H., Snyder, J. C., Cheever, F. S., and Murray, E. S.: J. Exper. Med., **91**:31, 1950; Hill, A. G. S., Deane, H. W., and Coons, A. H.: J. Exper. Med., **92**:35, 1950; Coons, A. H., Kaplan, M. H., and Deane, H. W.: J. Nat. Cancer Inst., **10**:1344, 1950.

28. Marshall, J. M.: J. Exper. Med., **94**:21, 1951.

29. Policard, A.: Compt. rend. Soc. de biol., **78**:32, 1915.

demonstrate urea at relatively low concentrations led Feyel[30] to believe that urea is not filtered through the glomeruli. Probably the best method is that of Oliver.[31]

Method

Fix small pieces of tissue in a mixture of 6 g. of xanthydrol, 35 ml. of alcohol, and 65 ml. of acetic acid for 6–12 hours. Heat the mixture gently and filter it before use. Dehydrate in alcohols, embed in paraffin. Counterstaining of the sections is permissible. The crystals are best recognized by their birefringence.

Uric Acid

Uric acid occurs in the tissues in the form of acid sodium urate, a substance of a very typical crystalline structure, sparingly soluble in water, insoluble in alcohol. Although somewhat soluble in dilute alkalis, it is practically insoluble in ammonia. Treatment with ammonia converts it into the corresponding ammonium salt, the crystalline structure of which is different from that of sodium urate.

Heavy-metal salts of uric acid are quite insoluble and can be converted into colored compounds;[32] however, such reactions lack specificity (phosphates and carbonates behave the same way). This applies also to most of the silver methods recommended for its demonstration.[33] However, uric acid is one of the few argentaffin substances (see p. 58) occurring in the animal body, and this property can be utilized for its identification. This has been done, although under nonoptimal conditions, by Gersh.[34] The following method gives very sharp and selective pictures.

30. Feyel, P.: Compt. rend. Soc. de biol., **114**:1155, 1933.
31. Oliver, J.: J. Exper. Med., **33**:177, 1921.
32. Saint-Hilaire, C.: Ztschr. f. physiol. Chem., **26**:102, 1898.
33. Courmont, J., and André, C.: J. de phys. et de path. gén., **7**:255, 1905; Tomita, W.: Tr. Jap. Path. Soc., **17**:190, 1927.
34. Gersh, I.: Anat. Rec., **58**:349, 1934.

Method

Fix tissues in 95–100 per cent alcohol. When mounting the sections on slides, float them on water for only a few seconds.

Carry slides through xylene and alcohols. From last alcohol, transfer them directly into methenamine-silver solution (see p. 60) buffered to pH ± 9 and prewarmed to 37° C. Keep them in the incubator for about 30 minutes or until urate shows up in a black shade. Rinse slides, remove unreacted silver with a dilute solution of Na thiosulfate. Gold toning is optional. Counterstain as desired.

Under low and medium powers the crystalline structure of the deposits appears to be preserved to perfection; under high power the slender needles are resolved into rows of fine black granules.

The only source of error is the presence of calcifications; they will be disturbing only if present in large masses. Otherwise, silver phosphate and carbonate are relatively easily soluble in methenamine and will be washed out of the tissue during incubation. In the case of massive deposits, some silver phosphate may remain undissolved. However, since it is not reduced to metallic silver (provided that the slide is not exposed to strong light), the rinse in thiosulfate will remove it. In case of doubt, treat section for 1 or 2 minutes with a 0.2–0.5 per cent solution of nitric or hydrochloric acid in absolute alcohol before transferring it to the silver solution (the acid must be washed off first with absolute alcohol). This treatment will completely eliminate calcifications. Urate deposits will remain undissolved, but their crystal structure will be somewhat distorted. On the other hand, urate deposits are removed readily by a dilute solution of lithium carbonate, which will leave calcifications intact. Confusion with melanin and premelanin, which are also argentaffin and will blacken under the same conditions, is not likely.

D. PROSTHETIC GROUPS

Phenolic Substances, Especially Polyphenols

The list of phenolic substances found in animal tissues includes tyrosine, adrenalin, certain propigments,[1] the enterochromaffin substance, the phenolic ketosteroids, and a few unidentified substances in lower species (oysters, cephalopods,[2] toads,[3] etc.[4]). Tyrosine was dealt with in the section on amino acids (p. 113). Phenolic ketosteroids have never been investigated histochemically except in a very uncritical way by Seeger.[5] This section will be devoted especially to adrenalin and the enterochromaffin substance. Before going into the specific histochemical properties of these substances, a few words must be said about color reactions for phenols.

There are a number of characteristic color reactions for phenols, some of which can be utilized for their histochemical identification. They will be enumerated in the order of their importance.

1) *The azo-coupling reaction.*—At an alkaline reaction, phenols will couple with diazonium salts to form intensely colored, water-insoluble azo dyes.[6] The shade of the dye depends on both the phenolic and the diazoic components; as a rule, a relatively low molecular weight of the components expresses itself in yellowish or orange shades; with increasing complexity of the molecule the shade will shift from orange to red, to purple, and finally to blue and black. However, besides molecular weight, structure is also an important determinant of shade; azo dyes produced from α- and β-

1. Lison, L.: Compt. rend. Soc. de biol., **106**:41, 1931.
2. Lison, L.: Histochimie animale, pp. 158–59 (Paris, 1936).
3. Shipley, P. G., and Wislocki, G. B.: Contrib. Embryol., **3**:73, 1915; Lison, L.: Compt. rend. Soc. de biol., **111**:657, 1932.
4. Lison, L.: Compt. rend. Soc. de biol., **112**:1237, 1933.
5. Seeger, P. G.: Ztschr. f. mikr.-anat. Forsch., **46**:153, 1939.
6. Saunders, K. H.: The aromatic diazo-compounds and their technical applications (London: Edward Arnold & Co., 1936); Pratt, L. S.: The chemistry and physics of organic pigments (New York: John Wiley & Sons, 1947).

naphthol, respectively, differ from one another sharply in color. The reaction is highly specific; under conditions of moderate alkalinity all phenols except those substituted in the para and both ortho positions will couple; the only other substances found in the tissues which will give a similar reaction are heterocyclic compounds (histidine; probably also proline). According to Pauly,[7] tryptophane does not couple; according to Danielli,[8] it does. In the writer's experiments no color was produced with tryptophane with five different diazonium salts. In general, the color of azo dyes resulting from amino acids is yellowish or pale orange-brownish.

2) *The indophenol reaction.*—This consists in the formation of bluish indophenol dyes when phenols unsubstituted in the para position are oxidized in the presence of aromatic amines. The reaction can be performed in a variety of ways. One of the simplest is Gibbs's method,[9] in which the amine and the oxidant are combined into a single substance (2,6-dichloro- or dibromo-quinonechloroimide); it also possesses the advantage of producing somewhat darker shades than the other variants.

3) *The ferric chloride reaction.*—Phenols give characteristic color reactions (green, blue, purple, depending on the nature of the phenol) with a dilute solution of ferric chloride. The shades produced in tissue sections are much too pale to be useful.

In addition to giving the reactions mentioned, diphenols are strong reducing agents. They will reduce an ammoniacal silver nitrate solution to metallic silver ("argentaffin reaction").[10, 11] By dichromates and iodates they are oxidized to quinones and other brownish, more or less insoluble, products of poorly known constitution which ultimately precipi-

7. Pauly, H.: Ztschr. f. physiol. Chem., **42**:508, 1904.
8. Danielli, J. F.: Cold Spring Harbor Symp. Quant. Biol., **14**:32, 1950.
9. Gibbs, H. D.: J. Biol. Chem., **72**:649, 1927.
10. Cordier, R.: Bull. d'histol. appliq. à la physiol., **4**:161, 1927.
11. Hamperl, H.: Virchows Arch. f. path. Anat., **286**:811, 1932.

tate ("chromaffin reaction");[12, 13] in the case of dichromates the precipitate will contain brownish chromium dioxide.[14] In the case of *o*- and *p*-diphenols, which are powerful reducers, these reactions are prompt; the reducing power of *m*-diphenols is much weaker, and the reactions take place over a period of hours rather than minutes. The corresponding aminophenols and diamines behave in an analogous way, but, since they are not known to occur in tissues, unless administered parenterally (arsphenamine),[15] their histochemical significance is very limited.

On account of the effects of fixation, the situation in tissues is somewhat more complicated than in the test tube. Nonprotein phenols appear to be soluble in alcohol; at least, they are not preserved by alcoholic fixatives. Formalin, on the other hand, undergoes condensation reactions (of the bakelite type) with phenols, resulting in the formation of highly insoluble resin-like substances[16] of varying molecular sizes. The identifying reactions of the condensation products may differ sharply from those of the parent phenols. This is easy to understand, since the hydroxymethyl groups entering the benzene ring have a tendency to occupy the coupling (ortho and para) positions; folding of the large polymer molecules formed later in the course of the reaction may cause instances of unpredictable steric hindrance or the approximation of reactive groups.

The changes in the chemical properties of phenols by formaldehyde can be studied conveniently by the use of Coujard sildes.[17] The phenols in question are dissolved in serum or dilute gelatin, and marks are made on slides with the solu-

12. Verne, J.: Bull. Soc. chim. biol., **5**:227, 1923.

13. Gérard, P., Cordier, R., and Lison, L.: Bull. d'histol. appliq. à la physiol., **7**:133, 1930.

14. Ogata, T., and Ogata, A.: Beitr. z. path. Anat. u. z. allg. Path., **71**:377, 1922–23.

15. Jancsó, N. von: Ztschr. f. d. ges. exper. Med., **61**:63, 1928; Jancsó, N. von: Arch. f. exper. Zellforsch., **6**:444, 1928.

16. Coujard, R.: Bull. d'histol. appliq. à la physiol., **20**:161, 1943.

17. Gomori, G.: Arch. Path., **45**:48, 1948.

tions. The slides are fixed in formaldehyde vapor and subsequently treated with various reagents. It will be observed that phenol, tyrosine, catechol, and hydroquinone lose their azo-coupling reactions after this treatment (or produce azo dyes so pale as to be indistinguishable from the shade of the control marks); the indophenol reaction of phenol and catechol is completely abolished. The chromaffin and argentaffin reactions of catechol and hydroquinone are greatly weakened. If formalin fixation is followed by treatment with 5 per cent potassium dichromate for 24 hours, the argentaffin reaction of these diphenols is abolished. On the other hand, resorcinol and phloroglucinol fully retain their azo-coupling ability and will produce azo dyes of brilliant shades; their indophenol reaction also remains unimpaired. They will show an intense chromaffin and argentaffin reaction; the latter is resistant to treatment with dichromate.

These observations are of great importance, as they show that in the case of phenolic substances the results of test-tube experiments cannot be used for their identification in fixed tissues.

Adrenalin

The adrenal medulla reduces alkaline silver solutions,[14] stains brown with dichromates,[18] gives azo dyes (of an inconspicuous ochre-yellow shade, suggestive of catechol derivatives) with diazonium compounds, shows a definite indophenol reaction and a greenish staining with ferric chloride solutions. There can be little doubt that all these reactions are due to the presence of adrenalin. However, they can be obtained with fresh material only; once the tissue is fixed either in formalin or in alcohol, they become negative. With alcohol, loss of reactivity is probably due to extraction of adrenalin from the tissue; with formalin, chemical changes induced in the molecule must be held responsible for the negative reactions.

For the histochemical demonstration of adrenalin, the tis-

18. Henle, J.: Ztschr. f. ration. Med., **24**:142, 1865.

sue should be fixed in Regaud's mixture (10 per cent formalin containing 5 per cent potassium dichromate). Mercury-containing fixatives, such as Zenker's fluid, give much poorer results. In the finished sections adrenalin-containing cells appear in a more or less dark-brown shade.

The argentaffin reaction (Ogata,[14] Baginski[19]) is not recommended; it does not give a sharp localization.

The Enterochromaffin (EC) Substance

The granules of the EC cells show a number of interesting staining reactions, some of which are capable of a chemical interpretation. All the reactions to be mentioned here can be elicited even after prolonged formalin fixation.

1. The granules are chromaffin.[20]
2. They are argentaffin,[21] even after fixation in dichromate-containing mixtures.
3. At an alkaline reaction they give intensely colored azo dyes with diazonium compounds.[22, 23]
4. They give a fairly intense indophenol reaction,[24] especially with Gibbs's reagent.
5. They reduce ferriferricyanide to Prussian blue.

In addition to these reactions, they stain intensely with some lake dyes (hematoxylin, gallocyanin, celestin blue, etc.) in the absence of metal salts.[25] The chemical explanation of this staining property is not clear. They are also stained by various silver-impregnation techniques, such as Bodian's[26] (this is not an argentaffin reaction; see p. 58).

On the basis of their chemical reactions, which are the same as those of adrenalin in the test tube (Verne[12]), Cor-

19. Baginski, S.: Bull. d'histol. appliq. à la physiol., **5**:129, 1928.
20. Heidenhain, R.: Arch. f. mikr. Anat., **6**:368, 1870.
21. Masson, P.: Compt. rend. Acad. sc., **158**:59, 1914.
22. Cordier, R., and Lison, L.: Bull. d'histol. appliq. à la physiol., **7**:140, 1930.
23. Lison, L.: Arch. de biol., **41**:343, 1931.
24. Jonnard, R.: J. de phys. et de path. gén., **32**:731, 1107, 1934.
25. Clara, M.: Ztschr. f. Zellforsch. u. mikr. Anat., **22**:318, 1934–35.
26. Dawson, A. B.: Anat. Rec., **91**:53, 1945.

dier[10] drew the conclusion that EC granules must contain an *o*- or a *p*-diphenol or aminophenol or diamine. Cordier and Lison,[22] in a later paper, found that all possibilities except that of an *o*-diphenol, with a short-chained substituent in one of the *p*-positions (there are two such positions on account of the two phenolic hydroxyls), can be ruled out. This view has received general acceptance, in spite of the fact that it does not explain either the discrepancies between the behavior of adrenalin and the EC substance or the slowness with which EC granules reduce alkaline silver solutions. Lison[1] thinks that adrenalin is simply not fixed by formalin, while the EC substance (or its protein matrix) is. It would be difficult to ascertain the correctness or incorrectness of this theory, the products of fixation being invisible. On the other hand, formalin-iodate or formalin-dichromate mixtures do fix both adrenalin and EC substance in the form of sharply localized brownish granules; yet adrenalin loses its typical reactions, while the EC granules retain them. Also, derivatives of catechol (adrenalin, 3,4-dihydroxyphenylalanine) and of hydroquinone (homogentisic acid) reduce silver solutions in a matter of seconds, while the EC granules require hours.

In Coujard slides the reactions of resorcinol are invariably identical with those of the EC granules,[17] including the shade of the azo dyes produced and even the staining by gallocyanin and celestin blue. On the basis of these observations, it appears safe to assume that the EC substance is a derivative of resorcinol and not of catechol.

The EC cells fluoresce intensely in ultraviolet light.[27] From his comparative studies of their fluorescence spectrum, Jacobson[28] came to the conclusion that these cells contain some derivative of pteridine. The evidence in favor of this assertion is convincing. However, his other theory, namely, that the typical reactions of the EC cells are due to a pteridine com-

27. Erös, G.: Zentralbl. f. allg. Path. u. path. Anat., **54**:385, 1932.
28. Jacobson, W.: J. Path. & Bact., **49**:1, 1939.

pound, cannot be accepted. Pteridine derivatives do not give a single one of the reactions of the EC cells in the Coujard experiment.

For the histochemical staining of the EC cells the argentaffin reaction, azo-coupling, and the indophenol reaction are recommended. With all techniques, prompt fixation of the tissue is important because the EC substance undergoes fairly rapid decomposition.

The reactions are quite intense with normal EC cells; in carcinoid tumors the intensity of staining is variable and may even be negative, depending on the degree of biochemical differentiation of the neoplastic cells.

1) *The argentaffin reaction.*—

Method

Fix tissues preferably in Bouin's fluid or in formalin. Other formalin-containing mixtures are also usable, but the contrast between the granules and the background is impaired by dichromates and mercury salts.

Treat sections for 10–60 minutes with Lugol's solution; decolorize with thiosulfate. This treatment helps to suppress the staining of the background. Wash slides thoroughly in distilled water. Incubate them for 12–24 hours at 37° C. either in methenamine-silver (p. 60), buffered at pH 8–8.5 with a borate buffer, or in Fontana's[29] solution (p. 60). Inspect the slides under the microscope at intervals; as soon as the EC cells appear black, remove them from the solution. Sections stained with methenamine-silver can be toned with gold chloride; gold toning is not advisable after Fontana's solution because it may cause considerable fading of the granules. Rinse sections in a dilute solution of Na thiosulfate, wash them under the tap, counterstain as desired, dehydrate, and mount.

The specificity of the method is satisfactory but, like that of all silver techniques, relative. On continued incubation

29. Fontana, A.: Dermat. Ztschr., **46**:291, 1925–26.

(over 24 hours) a number of additional structures will be stained; an early and intense blackening of eosinophilic and neutrophilic granules is especially conspicious.[11, 30] After 48 hours or more, practically the entire slide may turn solid black. If a darkish background develops on account of overstaining, differentiate slide as described under the Ag technique for glycogen and mucin (p. 64).

According to Burtner and Lillie,[31] the argentaffin reaction can be greatly accelerated by performing it at 60° C.

2) *The azo-coupling reaction.*—

Method

Dissolve about 50 mg. of either Red B Salt or Black B Salt or Garnet GBC Salt (chemical constitutions, p. 171) in about 10 ml. of cold water; add a few drops of a saturated solution of borax and pour the more or less turbid solution on the slide. Leave it on for 30–60 seconds; wash slide under the tap, counterstain lightly with hematoxylin, dehydrate, and mount. EC granules stain deep orange (Red B Salt), rusty red-brown (Black B Salt), or red (Garnet GBC Salt). The background is light yellow.

In order to obtain darker shades, Lison[23] recommends the use of tetrazotized diamines, in the hope that only one diazo group will couple with the tissue, and the second one can be coupled with naphthol. In this way intense purple or bluish shades could be produced. The method does work in practice, but the background becomes stained so heavily that the net gain in contrast is negligible. The two-step modification of Clara[32] (using unilaterally diazotized diamines and diazotizing the other side after coupling with the tissue has taken place) is not better than Lison's original method.

30. Cordier, R.: Arch. de biol., **36**:427, 1926.

31. Burtner, H. J., and Lillie, R. D.: Stain Technol., **24**:225, 1949.

32. Clara, M., and Canal, F.: Ztschr. f. Zellforsch. u. mikr. Anat., **15**:801, 1932; Clara, M.: Ztschr. f. wissensch. Mikr., **51**:316, 1934.

3) *The indophenol reaction.*—

This method gives distinctly less brilliant pictures than the previous ones but is quite specific.

Method

Dissolve about 50 mg. of 2,6-dibromoquinonechloroimide (Eastman No. 2304) or the corresponding dichloro-compound (Eastman No. 2483) in 5–10 ml. of alcohol, add 40–50 ml. of water and a few drops of a saturated solution of borax. Immerse slides for 10–15 minutes. The solution will turn a dark gray-brown. Remove slides, wash, and counterstain them with a red nuclear dye. Dehydrate and mount. EC granules stain in moderately intense shades of gray-blue.

Sulfhydryl (Thiol) Groups

Although sulfhydryl groups occurring in animal tissues are invariably carried by amino acids, their special biochemical significance warrants a discussion apart from the rest of the amino acids.

Of the sulfur-containing amino acids, only glutathion is uncombined and freely diffusible. The others (cysteine, methionine, etc.) are incorporated into protein molecules, share the solubility properties of proteins, and are precipitated by fixatives. Sulfhydryl compounds are fairly strong reducing agents and are transformed by oxidation into unreactive disulfides. Unless the tissues are examined fresh, most of their sulfhydryl groups will have undergone oxidation.

Since glutathion is diffusible, its exact histochemical localization is impossible, even if the tissue is fixed in a liquid which will precipitate it quantitatively (neutral formalin containing 1–2 per cent of cadmium acetate or lactate).[33] However, it is possible to localize sulfhydryl groups in proteins.

All the methods to be mentioned show the presence of

33. Binet, L., and Weller, G.: Bull. Soc. chim. biol., **16**:1284, 1934; Joyet-Lavergne, P.: Compt. rend. Soc. de biol., **128**:59, 1938.

thiol groups only. Disulfides do not react unless they are first reduced to thiols. The most effective reducers are sodium cyanide or sodium sulfite (5 per cent solutions; treat tissue for about 10 minutes).[34] It appears that trichloroacetic acid (2–20 per cent) or saturated ammonium sulfate also "reveal" or "unmask" disulfides; their mode of action is not clear.

The shades produced by the reagents are too pale to be observed in thin sections; thick frozen sections or teased preparations of fresh tissues are preferred. Fixation in formalin-saline or alcohol is permissible.

1) *The nitroprusside reaction (Buffa).*[35]—Alkalized nitroprusside (about 5 per cent nitroprusside with 1–2 per cent ammonia added) produces a fleeting purple coloration with –SH groups. If the tissue is dipped for a few seconds in a 5 per cent solution of zinc acetate and then transferred to the reagent, the color will be stable enough to permit dehydration and mounting of the tissue.[36]

This reaction appears to be specific. The only other substance giving a similar reaction is creatinine; it is washed out of tissues by a short rinse.

2) *The ferriferricyanide method (Chèvremont and Frederic).*[37]—If tissues are treated with a freshly prepared solution containing about 0.2 per cent each of potassium ferricyanide and ferric ammonium citrate, with a few drops of dilute acetic acid added, –SH groups will reduce the ferricyanide and produce a precipitate of Prussian blue. The specificity of this method is limited; almost any reducing substance will be stained blue more or less promptly.

34. Joyet-Lavergne, P.: Bull. d'histol. appliq. à la physiol., **5**:331, 1928; Joyet-Lavergne, P.: Compt. rend. Soc. de biol., **98**:658, 1928; Serra, J. A.: Stain Technol., **21**:5, 1946.

35. Buffa, E.: J. de physiol. et path. gén., **6**:645, 1904.

36. Giroud, A., and Bulliard, H.: Bull. Soc. chim. biol., **14**:278, 1932; Giroud, A., and Bulliard, H.: Protoplasma, **19**:381, 1933; Giroud, A., and Bulliard, H.: Bull. d'histol. appliq. à la physiol., **11**:169, 1934; Giroud, A., and Bulliard, H.: Arch. d'anat. micr., **31**:271, 1935.

37. Chèvremont, M., and Frederic, J.: Arch. de biol., **54**:589, 1943.

3) *Bennett's method.*[38]—This method is based on the well-known reaction between sulfhydryl groups and chloromercuriphenol. The reagent is *p*-chloromercuriphenylazo-β-naphthol, which is a poorly soluble red dyestuff. It will stain proteins containing –SH groups in a very specific way but in a rather pale reddish shade.

It would be interesting to synthesize chloromercuri-α-naphthol, which should also combine with sulfhydryl groups. In a second step, it could be coupled with a suitable diazonium salt (e.g., Blue B Salt) to yield a very dark purple-black azo dye.

E. VARIOUS UNCLASSIFIED ORGANIC SUBSTANCES

Flavoproteins

Chèvremont and Comhaire[1] have devised a method for the histochemical demonstration of riboflavin, based on the fact that riboflavin, if first reduced to leucoflavin, will reoxidize in air to red rhodoflavin. Since frozen sections of formalin-fixed material are used, only protein-bound riboflavin will be demonstrated.

Method

Move frozen sections around in 1–2 per cent HCl, containing enough zinc dust to keep it bubbling, for about 30 minutes. Rinse sections in distilled water and expose them to air for a few hours in a shallow layer of water. Mount in glycerin-gelatin. Flavoproteins are stained red.

This is an untested method. Sodium hydrosulfite ($Na_2S_2O_4$) would be a simpler reducer than nascent hydrogen and just as effective.

There is a possibility that flavoproteins could be localized by their green fluorescence. The distinctive feature is the

38. Bennett, H. S.: Anat. Rec., **100**:640, 1948; Bennett, H. S., and Yphantis, D. A.: J. Am. Chem. Soc., **70**:3522, 1948; Mescon, H., and Flesch, P.: J. Nat. Cancer Inst., **10**:1370, 1950.

1. Chèvremont, M., and Comhaire, S.: Arch. f. exper. Zellforsch., **22**:658, 1939.

immediate disappearance of fluoresence on the addition of hydrosulfite to the mounting medium.

Lewisite

Lewisite, a war gas, is a mixture of chlorovinylarsins. In an alkaline medium it decomposes to yield acetylene; the latter will precipitate cuprous ions in the form of red copper carbide. This reaction is utilized for the histochemical demonstration of lewisite.

Method[2]

Prepare three stock solutions: A, an 8 per cent solution of cupric sulfate in distilled water; B, a 20 per cent solution of sodium sulfite in distilled water; C, a solution of 50 g. of sodium thiosulfate and 20 g. of sodium hydroxide in 80 ml. of distilled water to which 1 ml. of piperidine has been added. The latter enhances the red shade of the precipitate. For use, mix 10 ml. each of solutions A and B; when clear, add 10 ml. of solution C. The mixture is unstable and can be used for only about 1 hour.

Dip frozen sections of fresh tissue in the reagent for a few seconds; wash them, dehydrate in alcohol, and mount in balsam. Counterstaining is not recommended. This is an untested method.

"Mustard" (Dichlorodiethylsulfide)

Gold chloride reacts with mustard to yield a bright yellow complex, which can be reduced to black metallic Au.

Method[3]

Immerse frozen sections of fresh tissues in a 1 per cent solution of gold chloride for 2–3 minutes. Mount on a slide, flood with 5 per cent sodium hydroxide for 30 seconds. Wash, dehydrate, and mount in balsam. Shades of black and

2. Ferguson, R. L., and Silver, S. D.: Am. J. Clin. Path., **17**:37, 1947.
3. Silver, S. D., and Ferguson, R. L.: Am. J. Clin. Path., **17**:39, 1947.

purple-black indicate the presence of mustard. This is an untested method.

F. PIGMENTS

Pigments will be defined as substances visible in unstained preparations by virtue of their own color. They form an extremely heterogeneous group as to both their origin and their chemical nature. Formalin pigment, for example, is an artifact, not originally present in the tissues but produced by the action of formaldehyde on hemoglobin. Some of the pigments are entirely foreign to the organism, introduced accidentally ("exogenous" pigments, such as carbon and various metals); others are the products of normal or pathological metabolic processes within the organism ("endogenous" pigments).

The number of colored substances found in various animal and plant species is very large, and but a small fraction of them have been identified chemically. In this section only such pigments as are encountered in tissue sections of man and the more common laboratory animals will be dealt with.

I. Formalin pigment[4] occurs in tissues fixed in acid formalin or formalin-containing mixtures; it is found mainly in and around larger collections of blood. It forms brown-black granules of a crystalline structure. Two methods of removal are recommended.

1) *Barrett's method.*[5]—Treat the section for 10 minutes to 2 hours with a saturated solution of picric acid in alcohol.

2) *Kardasewitsch's method.*[6]—Treat the section for 1–2 hours with a 2–3 per cent solution of concentrated ammonia water in 70–80 per cent alcohol.

The formation of formalin pigment can be prevented by using a formaldehyde solution buffered to pH 6–7 with M/15 phosphate.

4. Lillie, R. D., and Hershberger, L. R.: Bull. Internat. A. M. Mus., **27**:136, 1947; Hershberger, L. R., and Lillie, R. D.: Bull. Internat. A. M. Mus., **27**:162, 1947.

5. Barrett, A. M.: J. Path. & Bact., **56**:135, 1944.

6. Kardasewitsch, B.: Ztschr. f. wissensch. Mikr., **42**:322, 1925.

II. The exogenous pigments are mainly carbon and various metals or metal-protein compounds, possibly also sulfides. Carbon can be recognized by its opaque blackness and by its absolute resistance to all bleaching agents and solvents. Metallic pigments have been discussed in the section on "Metallic elements" (p. 29).

III. The endogenous pigments fall into several classes according to their origin and chemical nature. Some of the individual pigments are chemically well-defined entities; others are a conglomeration of a number of related substances which probably represent successive stages in the evolution of an ultimate colored substance. The most important endogenous pigments will be divided into three groups:

1. Hematogenous pigments include hemoglobin and its degradation products, some of which contain ferric iron and some of which are iron-free.

2. Phenolic pigments are formed by the oxidation and polymerization of catechol and hydroquinone (possibly also tyrosine) derivatives.

3. Lipogenous pigments result from the oxidation and polymerization of unsaturated fatty acids.

Other chemically well-defined pigments, such as porphyrins and carotenoids, very seldom occur in the tissues of higher species in concentrations high enough to be visible under the microscope. Crustacean tissues, on the other hand, may be very rich in carotenoids.

1) *Hematogenous pigments.*—

A) *Iron pigments.*—*a*) Hemoglobin is demonstrated by its peroxidase action (p. 162). It should be remarked that unaltered hemoglobin is best identified by the zinc-leuco method, but its immediate degradation products, such as those seen in renal tubules 1 or 2 days after a hemolytic reaction, may not stain at all. They can be demonstrated by the benzidine method. It can be shown in test-tube experiments that the recolorization of zinc-leuco dyes by peroxide is a true enzymatic reaction requiring hemoglobin itself, whereas

the benzidine reaction can be catalyzed also by nonprotein heme compounds.

b) Hemosiderin is a yellowish, brownish, or greenish-brown granular pigment which resists the bleaching action of hydrogen peroxide and of permanganate and is not argentaffin. It does not dissolve in dilute acids or alkalis. It can be identified by the Prussian blue test (p. 38).

c) Malaria pigment is an amorphous brown-black granular substance, soluble in dilute alcoholic alkalis or Barrett's solution (p. 132); it is bleached by 3 per cent hydrogen peroxide within 2 hours. It has repeatedly been identified as hematin.[7] Naturally, the pigment will not give a direct reaction for iron. After destroying the organic matter with chlorine, hydrogen peroxide, or alkali, a positive Prussian blue reaction can be obtained.[8] It is not possible to differentiate malaria pigment from formalin pigment histochemically. In diagnostic cases, the formation of formalin pigment must be prevented by proper fixation.

B) *Iron-free pigments.*—Bile pigments appear under the microscope as yellowish-green, more or less coarse granules which are not bleached by hydrogen peroxide or by permanganate and are not argentaffin. They are slowly converted by oxidants (hydrogen peroxide, Lugol's solution,[9] nitrous acid,[10] dichromates, etc.) into green biliverdin. Hematoidin is chemically identical with bilirubin and shares its reactions.

2) *Phenolic pigments.*—

A) Melanin is an oxidation-polymerization product of dioxyphenylalanine and possibly also of tyrosine.[11] It forms yellowish-brown or grayish to almost jet-black granules, in-

7. Sinton, J. A., and Ghosh, B. N.: Rec. Malaria Survey India, **4**:205, 1934; Morrison, D. B., and Anderson, W. A. D.: Pub. Health Rep., **57**:90, 1942; Devine, J., and Fulton, J. D.: Ann. Trop. Med., **36**:167, 1942.

8. Kósa, M.: Virchows Arch. f. path. Anat., **258**:186, 1925; Okamoto, K.: Taishitzu Gaku Zasshi, **13**:55, 1944.

9. Stein, J.: Compt. rend. Soc. de biol., **120**:1137, 1925.

10. Okamoto, K., Sengoku, M., and Hirotani, N.: Taishitzu Gaku Zasshi, **14**:30, 1948.

11. Hasebroek, K.: Fermentforsch., **5**:1, 1922; Sato, K., and Brecher, L.: Arch. f. mikr. Anat., **104**:649, 1925.

soluble in dilute acids and alkalis and promptly bleached by acidified permanganate and slowly (in several hours to days) by hydrogen peroxide. It is argentaffin in that it will reduce alkaline silver solutions (Foot's or alkaline silver-methenamine, p. 60) in about 3–12 hours. It does not give the Prussian blue reaction.

B) The pigment of ochronosis derives from an unidentified derivative of hydroquinone (possibly homogentisic acid). It imparts a homogeneous yellow-brown to blackish hue to certain tissues (cartilage, degenerated elastic fibers, etc.). It is not argentaffin. Its most typical tinctorial property is its very intense, almost black, staining with cresyl violet.[12]

3) *Lipogenic pigments.*—

Iron-free, brownish pigments, stainable by fat dyes (to some extent even after paraffin-embedding), rather resistant to dilute acids and alkalis and to bleaching by oxidants, have been described under various names (chromolipoid,[13] hemofuscin,[14] lipofuscin,[15] "Abnutzungspigment,"[16] pigment of wear and tear, luteolipin,[17] ceroid,[18] cytolipochrome,[19] etc.). Their other properties include a brownish fluorescence, staining by basic aniline dyes, especially fuchsin,[20] increase in basophilia after oxidation by permanganate,[21] metachromasia with methyl green,[22, 23] a positive Schiff reaction after periodate treatment,[17, 24] peroxide-like effects, etc.[22, 25] Whether

12. Friedrich, H., and Nikolowski, W.: Arch. f. Dermat. u. Syph., **192**:273, 1951.
13. Ciaccio, C.: Biochem. Ztschr., **69**:313, 1915.
14. Recklinghausen, F. D. von: Berl. klin. Wchnschr., **26**:925, 1889.
15. Borst, M.: Pathologische Histologie (Leipzig: F. C. W. Vogel, 1922).
16. Lubarsch, O.: Zentralbl. f. allg. Path. u. path. Anat., **13**:881, 1902.
17. Rossman, I.: Carnegie Inst. Washington Publ., **541**:97, 1942.
18. Lillie, R. D., Ashburn, L. L., Sebrell, W. H., Daft, F. S., and Lowry, J. V.: Pub. Health Rep., **57**:502, 1942.
19. Gillman, J., and Gillman, T.: Am. J. Path., **40**:239, 1945.
20. Mallory, F. B.: Pathological technique (Philadelphia: W. B. Saunders Co., 1938).
21. Endicott, K. M., and Lillie, R. D.: Am. J. Path., **20**:149, 1944.
22. György, P.: Am. J. Clin. Path., **14**:67, 1944.
23. Popper, H., György, P., and Goldblatt, H.: Arch. Path., **37**:161, 1944.
24. Elftman, H., Kaunitz, H., and Slanetz, C. A.: Ann. New York Acad. Sc., **52**:72, 1949.

or not all or most of these characteristics apply to all forms of the pigment cannot be decided, because there are only a few thorough studies available; in most reports only a few of the properties are mentioned. In the writer's experience the type and duration of fixation markedly affect staining properties. Dichromate or moderately prolonged fixation in formalin tends to enhance basophilia, the Schiff reaction, acid-fastness, and affinity to fat stains after paraffin-embedding, whereas alcohol or short fixation in formalin has the opposite effect. The pigment granules in one section, even within one cell, may exhibit considerable variation in staining intensity. Minor species differences have been noted by Lee.[26]

It appears that all the pigments in this group are of an essentially similar nature. They all derive from the oxidation and polymerization of unsaturated fatty acids and represent various stages of one process. Staining reactions similar to the ones mentioned will develop in droplets of unsaturated fat injected into the tissues[27] and even in fats allowed to stand in air.[13, 28] Since the chemistry of the oxidation of unsaturated fats is very incompletely understood, it is impossible to specify just what varying degrees of such staining properties as basophilia or acid-fastness could mean. However, since all transitions from one extreme to the other may be observed in a single slide, it is doubtful whether any classification within the group is biologically meaningful. Ceroid, for example, is probably only a late product in the maturation of lipogenic pigments in general, although the process may have been accelerated and have actually progressed beyond the normal average.

Gillman and Gillman's cytosiderin[19] appears to be a mixture of hemosiderin with lipogenous pigment.

25. Glavind, J., Granados, H., Hartmann, S., and Dam, H.: Experentia, **5**:84, 1949.

26. Lee, C. S.: J. Nat. Cancer Inst., **11**:339, 1950.

27. Hass, G. M.: Arch. Path., **27**:15, 1939; Endicott, K. M.: Arch. Path., **37**:49, 1944.

28. Smith, J. L.: J. Path. & Bact., **11**:415, 1906.

CHAPTER VIII

ENZYMES

The microscopic identification and localization of enzymes in tissue sections presents the youngest offshoot of histochemistry. True, a few reactions purporting to reveal enzymes were described a long time ago; however, some of them were shown later to be definitely nonenzymatic, and the enzymatic nature of others is still debatable.

At the present time only a very limited number of enzymes can be demonstrated histochemically. The vast majority of enzymes are too labile to resist the manipulations necessary in histological technique. Others are resistant enough, but, so far, no reactions suitable for histochemical application have been devised for their demonstration.

Fortunately, a fair number of enzymes, mainly of the hydrolytic series, resist a certain amount of physical and chemical treatment and will tolerate histotechnical manipulations without undue loss of activity. Moreover, they catalyze reactions which yield insoluble precipitates, suitable for microscopic localization.

Whether or not an enzyme is sufficiently resistant to be demonstrated histochemically can be determined either by (1) exposing Coujard slides, marked with serial dilutions of an active extract, to the physical and chemical agents used in the histological routine (fixatives, dehydrating and clearing agents, hot paraffin, etc.) or by (2) chemical assay of the activity of tissue samples in the fresh state and after treatment with the agents mentioned. Experience shows that fair histochemical results can be obtained even if as much as 90 per cent of the enzyme is destroyed in the course of manipulations. Of course, in such cases the microscopic picture cannot be expected to reveal the full extent of activity,

because at sites of low enzyme concentration activity is likely to drop below the threshold of sensitivity of the method.

PREPARATION OF TISSUES FOR ENZYMATIC REACTIONS

The tissue need not be absolutely fresh, but refrigeration is advisable whenever processing cannot be prompt. In most cases satisfactory results are obtained with tissues preserved in the icebox for several (often up to 48) hours, although the pictures may not be quite so sharp as with fresh tissue.

If fixation is not permissible, fresh-frozen sections must be used. In such cases it is imperative to keep the enzyme undissolved; otherwise correct localization is impossible unless the enzyme happens to be insoluble. The simplest way to achieve this is to avoid contact with water or with dilute saline solutions and to use strong solutions of ammonium sulfate (½ saturated or better) up to the point in the procedure at which the localizing precipitate has been obtained.

It is very likely that a rapid freezing-cutting method, such as that of Adamstone and Taylor[1] or the simpler original procedure of Schultz-Brauns,[2] followed by a brief fixation in acetone, would give good results with many enzymes.

The optimal method for most enzymes is probably the freezing-drying technique. Unfortunately, most workers do not possess the equipment and must depend on simpler methods, similar to those used in the average laboratory of histology or pathology. It is to such methods that this chapter will be devoted.

Fixation.—As a rule, the best fixative for all enzymes is chilled acetone, which, of all fixatives, causes the least inactivation. A bottle of acetone should be kept in the icebox for routine use. Cytological details are not so good as one would like them to be, but they are satisfactory for most purposes if the slices are thin enough (not over 3 mm. in thickness). It is advisable to chill (not freeze) the tissue itself before

1. Adamstone, F. B., and Taylor, A. B.: Stain Technol., **23**:109, 1948.
2. Schultz-Brauns, O.: Klin. Wchnschr., **10**:113, 1931.

throwing it into the cold fixative; many shrinkage artifacts can be avoided in this way. Acetone is especially recommended for acid phosphatase. For other enzymes, cold ethyl alcohol (90–100 per cent) is equally satisfactory and actually preferable because it gives a better cytological fixation and the tissue is easier to handle. A compromise fixative consisting of equal volumes of acetone and alcohol will give excellent results in the overwhelming majority of cases. Methyl alcohol is entirely unsuitable as a fixative, since it destroys most enzymes. Most hydrolytic enzymes are reasonably resistant to formalin[1] and can be fixed in 10 per cent formalin[3] (preferably adjusted to pH 6–6.5 with a small amount of phosphate buffer). Again, fixation in the icebox is recommended. Formalin-fixed tissues should be cut frozen; embedding usually gives very poor results, even if the formalin is washed out carefully. Exceptions will be mentioned under the individual enzymes.

Dehydration and embedding.—The successful use of frozen sections has been reported repeatedly, especially after formalin fixation. Alcohol- and, even more, acetone-fixed tissues cut poorly by the freezing method. In addition, the enzymes are not always irreversibly precipitated by these fixatives. In general, embedding is preferable whenever feasible.

For dehydration, alcohol or acetone can be used. For best results, dehydration should be carried out at icebox temperature. After complete dehydration the pieces can be embedded though benzene or chloroform into paraffin. Regular celloidin-embedding, although it may yield good results in some cases, is not recommended because it requires too long an exposure to alcohol-ether; and, in addition, the dilute alcohol in which celloidin blocks are stored is quite harmful to enzymes. Semi-embedding in dilute celloidin has been mentioned previously (p. 15).

Acetone-fixed tissues have a tendency to crumble during

3. Seligman, A. M., Chauncey, H. H., and Nachlas, M. M.: Stain Technol., **26**:19, 1951.

cutting and to disintegrate when floated on water. The remedy is infiltration of the blocks with thin celloidin before paraffin-embedding.

The temperature of the paraffin oven should not exceed 56° C., and the tissues should not be exposed to this temperature for longer than 1½ hours. Embedding can be hastened by the use of reduced pressure.[4] A piece of rubber tubing, connected through a safety bottle to a water pump and introduced into the oven through its ventilating opening, will serve as a simple suction apparatus. The melted paraffin is kept in a wide-mouthed bottle which has a tight-fitting stopper, with a piece of glass tubing in its single perforation. The end of the rubber tubing is attached to the glass. The bulk of benzene or chloroform is removed by the first change of paraffin in an open dish (about 15–20 minutes). The pieces are now transferred to the suction bottle, and the vacuum is turned on gradually. Bubbling, quite lively at the start, soon decreases and ceases altogether in about 30 minutes. A third change of paraffin in an open dish (about 10–15 minutes) completes infiltration, and the pieces are then ready to be embedded.

The following schedule of fixation and embedding is suggested for all enzymes unless otherwise specified in the text:

1. Chill tissue for 15–30 minutes in the icebox. Fix slices not thicker than 3 mm. in chilled acetone for 24 hours. It is advisable to trim the pieces even thinner with a razor blade as soon as they have gained some consistency (2–3 hours).
2. Dehydrate in two or three changes of absolute acetone or alcohol, about 12 hours each.
3. Transfer pieces to a mixture of equal volumes of alcohol and ether for a few hours.
4. Transfer to a 2–3 per cent solution of celloidin (collodion, U.S.P., diluted with ½ to 1 volume of alcohol-ether mixture) for 12–24 hours.
5. Drain pieces rapidly and carry them through two changes of chloroform, ½–1 hour each.

4. Gomori, G.: Am. J. Clin. Path., **16**:347, 1946.

6. Embed in paraffin under reduced pressure, as described above.

Steps 1–4 should be performed at icebox temperature.

In the case of alcohol or acetone-alcohol fixation, steps 3 and 4 can be omitted; in step 5 benzene may be used instead of chloroform.

Sections are cut at 3–8 μ, floated on lukewarm water, mounted with albumin-glycerol, and dried. The dried sections should be melted in the paraffin oven (5–10 minutes). The coating of paraffin they acquire by this treatment appears to have a protective action against atmospheric influences (oxygen, moisture). Unmelted sections may lose most of their activity in a matter of a few weeks or months, whereas melted ones, just like uncut paraffin blocks, remain virtually unchanged for many years.

For use, the slides are carried through xylene and alcohols to water. It may be advisable to protect the tissue against loss of enzyme with a thin layer of collodion. This can be done by flooding the slide (after the second alcohol) with dilute (about 0.5 per cent) collodion in alcohol-ether, shaking off the excess, and hardening the membrane for a minute or so in 80–95 per cent alcohol. Some substrates do not pass the membrane readily. In such cases, protection with collodion should be omitted, and even the collodion from the embedding procedure should be removed by a short rinse in alcohol-ether or acetone.

HISTOCHEMICAL REACTIONS FOR ENZYMES

Sometimes the primary product of the reaction is an insoluble dye (e.g., red formazan from triphenyltetrazolium chloride) or a soluble one (e.g., acid aniline dyes from the corresponding leuco-dyes), permitting immediate visualization of sites of activity. Soluble dyes, as a rule, are much less reliable as far as correct localization is concerned, because they will stain the true sites of liberation only if they have an affinity to some structure present locally; otherwise they may diffuse away and may even stain distant and inactive structures for which they do possess an affinity.

In other cases, including all hydrolytic enzymes, the product of enzymatic activity is a colorless and soluble substance (ions, phenolic compounds) and must be precipitated in a second step by a suitable reagent, added to the incubating mixture. If correct localization and high sensitivity are to be achieved, precipitation must be very prompt (of the velocity of ionic reactions) in order to bind the reaction product as fast as it is liberated, and the precipitate must be exceedingly insoluble. The less perfectly these conditions are satisfied, the less sensitive the method and the poorer the localization of activity will be.

These important principles will require a somewhat more detailed consideration.

No substance is totally insoluble. If an "insoluble" compound is made from its components in a solution, it will precipitate only if and when its solubility (or, in the case of a salt, solubility product) is exceeded. Constancy of results, maximum sensitivity and correct localization in a histochemical experiment can be attained only if the velocity of precipitation is almost infinite and the precipitate is practically insoluble. These two aspects do not necessarily run parallel; even in the case of highly insoluble precipitates a transient phase of supersaturation or colloidal state may occur. Fortunately, this is very rarely observed in practice. In the optimal case all the reaction product will be precipitated immediately and at the exact site where it is formed. Otherwise, some of it will diffuse away from the primary sites of formation before it can be precipitated and raise the concentration in the ambient fluid. In unfavorable cases very little or even none will precipitate locally, in spite of high enzymatic activity; practically all the reaction product will be washed away and contribute to the saturation of the medium, first only around centers of high activity and later everywhere. The difference between the rates of hydrolysis and of loss by diffusion must have a minimum absolute value, proportional to the solubility of the precipitate, for a local precipitation to

occur. This fact explains the interesting observation of "all-or-none" effects[5, 6] in enzymatic histochemistry: either the minimum value is exceeded, and a local precipitation will be obtained; or it is not, and the reaction will be negative. In the borderline region, a minimal change in conditions may cause very marked differences in the extent of the reaction. Even serial sections incubated together in the same Coplin jar may exhibit wide variations in the pattern of distribution, on account of random convection currents in the substrate mixture, causing a temporary imbalance of concentrations and of temperature. Such chance variations may easily be misinterpreted as significant.

False negative reactions at the sites of enzymatic activity are only one type of error due to too soluble a precipitate. A different kind of error may be caused by the diffusion of the undeposited reaction product into the incubating medium. If and when the point of saturation is reached, the solute will settle out indiscriminately all over the slide. Such precipitates are usually coarsely crystalline, easy to recognize, and not likely to be mistaken for the true localization of the enzymatic reaction. However, there exists another type of artifact that is easily confused with a true reaction. Certain structures may possess an affinity for small molecules such as are produced by enzymatic hydrolysis and may adsorb them selectively, even from incompletely saturated solutions. A biological example of this is the *in vitro* calcification of cartilage in near-saturated solutions of calcium phosphate. A similar phenomenon is observed under the conditions of the histochemical method for alkaline phosphatase. Some structures, such as bone matrix and cell nuclei, themselves enzymatically inactive, readily adsorb calcium phosphate produced by enzymatic hydrolysis elsewhere, at active parts of the section.[6] As can be expected, such artifacts will occur preferentially in the immediate vicinity of highly active cen-

5. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **70**:7, 1949; Gomori, G.: Ann. New York Acad. Sc., **50**:968, 1950.

6. Gomori, G.: J. Lab. & Clin. Med., **35**:802, 1950.

ters[7–10] where a transient high concentration of phosphate ions may prevail for relatively long periods. This type of artifact will be accentuated on prolonged incubation. The secondary staining of nuclei, themselves inactive, is a typical example of a false localization,[11, 12] that is, a positive reaction due to enzyme contained in the tissue proper but appearing at sites other than those of the true primary microscopic localization.

In a recent paper Johansen and Linderstrøm-Lang[13] give a quantitative evaluation of diffusion artifacts in the calcium-cobalt technique for alkaline phosphatase. They come to the conclusions that (1) calcium phosphate has a great tendency to form supersaturated solutions, and (2) the time required for the phosphate concentration to reach the critical level may vary from approximately 0.1 to 2.5 seconds. This time is ample to allow gross diffusion into the neighboring areas, where conditions may be more favorable for precipitation (the presence of preformed crystal nuclei; special chemical or physical configuration of cell structures). Therefore, it is very likely that calcium phosphate will precipitate at such preferential sites rather than in centers of high enzymatic activity.

While the possibility of diffusion artifacts cannot be denied, the conclusions of Johansen and Linderstrøm-Lang are based on purely theoretical assumptions, some of which are not borne out by experimental facts and which make the situation look much worse than it actually is. For instance, the authors do not take into account the results of experiments showing that diffusion artifacts occur mainly under

7. Danielli, J. F.: J. Exper. Biol., **22**:110, 1946.
8. Jacoby, F., and Martin, B. F.: Nature, **163**:875, 1949.
9. Martin, B. F., and Jacoby, F.: J. Anat., **83**:351, 1949.
10. Feigin, I., Wolf, A., and Kabat, E. A.: Am. J. Path., **26**:647, 1950.
11. Kroon, D. B.: Acta endocrinol., **2**:227, 1949; Doyle, W. L.: Am. J. Anat., **87**:79, 1950; Cleland, K. W.: Proc. Linnean Soc. New South Wales, **75**:54, 1950; Novikoff, A. B.: Science, **113**:320, 1951.
12. Gomori, G.: J. Lab. & Clin. Med., **37**:526, 1951.
13. Johansen, G., and Linderstrøm-Lang, K.: Acta chem. Scandinav., **5**:965, 1951.

nonoptimal conditions of incubation, such as too low pH values or Ca ion concentration. As the pH and Ca ion concentration are raised, the localization becomes progressively sharper, and the stained areas shrink concentrically. Actually, under optimal conditions (p. 184) and an incubation time not exceeding 1½ hours, the diffusion artifacts described by Danielli[7] and by Jacoby and Martin[8, 9] cannot be reproduced.[14] This observation, together with the facts that (1) artificial gradual supersaturation of the substrate mixture or the diffuse impregnation of the section with enzyme produce pictures entirely different form those obtained by the regular method and (2) the localization of activity is the same with the calcium-cobalt and the azo dye methods, does not support the theory of the importance of false secondary localizations. Furthermore, the thesis that phosphatase is distributed evenly (or at random) among all cells or even within one cell body cannot be accepted. Comparison with quantitative Coujard slides clearly shows that in many (if not most) cases the enzyme is concentrated in very small, discontinuous spots, the activity of which may exceed the average value for the whole tissue by a factor of the order of 100. Activities as high as 700 Bodansky units per gram of active structure (as against the average value of 1 unit, assumed by the authors) have been observed.[15] On the basis of the facts mentioned, it is safe to assume that the highest intensity of the histochemical reaction coincides with centers of enzymatic activity. Further experiments will have to decide whether the diffusion artifacts persisting in spite of optimal conditions are within or beyond the resolving power of high-power dry objectives.

It must be mentioned here that diffusion of the enzyme itself has been blamed for false localizations.[10, 16, 17] Diffusion

14. Gomori, G.: Unpublished.
15. Gomori, G.: Exper. Cell Research, **1**:33, 1950.
16. Danielli, J. F.: Nature, **165**:762, 1950.
17. Hébert, S.: Arch. de biol., **61**:235, 1950; Yokoyama, H. O., Stowell, R. E., and Tsuboi, K. K.: J. Nat. Cancer Inst., **10**:1367, 1950; Yokoyama, H. O., Stowell, R. E., and Mathews, R. M.: Anat. Rec., **109**:139, 1951.

of the enzyme from sections of embedded material must be considered unlikely,[12, 18] especially if collodion protection is used. At least, solutions in which a larger number of slides, carrying sections of highly active material, are soaked for hours, and even days, never acquire any demonstrable activity. However, enzymes may be lost by diffusion from unfixed or poorly fixed tissues (frozen-dried material) unless the proper precautions are taken (p. 12). The enzyme dissolved in the incubating medium may slowly decompose the substrate, cause its gradual saturation, and produce artifacts of the type described in the preceding passage. Theoretically, the enzyme itself may even be adsorbed in a secondary way on various structures; however, it has been shown experimentally that such an adsorption can occur only under very abnormal conditions,[12] not in the least likely to be met with in practice.

In the case of precipitates which are not extremely insoluble, diffusion currents simply wash away part of the reaction product and prevent its precipitation. On the other hand, the relative sluggishness of these currents may cause a somewhat different type of artifact if the precipitate is very insoluble, mainly in the case of some azo dyes but possibly even in the case of phosphates. Under such conditions the reagent may become exhausted in the vicinity of centers of very high activity more quickly than it can be supplied by diffusion from near by. With this lack of precipitant, the product of hydrolysis will remain unbound and free to diffuse until it encounters a fresh supply of reagent some distance away. This will result in too little staining at the sites of true activity and an irregular precipitate of azo dye (or, in the case of Ca phosphate, staining of nuclei) around them. This type of artifact can be prevented by agitating the slide vigorously during incubation, and so hastening the interchange of fluid around the tissue.

18. Doyle, W. L.: Quantitative aspects of the histochemistry of phosphatases. In Symposium on cytology (East Lansing: Michigan State College Press, 1951).

While it may prove impossible to prevent artifacts completely and in every case, every effort must be made to reduce their occurrence. It is imperative to insure the promptest possible precipitation of reaction products formed. This can be done mainly by increasing the insolubility of the precipitate, e.g., by low ionic strength, an appropriate pH, and a high concentration of the precipitating ion. In addition, incubation should not be prolonged beyond the optimum.[16] The details of these measures will be given with the individual techniques. Presaturation of the incubating medium with the precipitate to be formed is also practiced, but this procedure is not very reliable. If saturation is not complete, it is of little help; if it is, the slightest evaporation of the medium or change in its temperature may lead to supersaturation, with a resulting indiscriminate precipitation. A high rate of enzymatic activity is also important because of the critical nature of the difference between rates of hydrolysis and of diffusion. Enzymatic activity can be enhanced by a high substrate concentration, the use of activators and incubation at the optimal pH. Inhibitors should not be employed except for specific purposes.

It is obvious that slow reactions for the precipitation of the primary product of enzymatic activity cannot localize correctly, no matter how insoluble the final precipitate may be. For instance, indoxyl liberated enzymatically can be visualized by oxidation into insoluble indigo. The picture obtained, however, can serve only as a rough indication of the true distribution of activity, since the process of oxidation is a relatively slow one.

The precipitate obtained in the course of enzymatic reactions is often colorless and invisible under the microscope. In such cases it must be transformed into a colored substance. The choice of the appropriate chemical procedure to accomplish this will vary with the nature of the primary precipitate. If the steps are well planned, there is little danger of producing spurious secondary localizations, because the

reactions take place without any significant solubilization of the precipitate.

False positive reactions, i.e., reactions misleadingly similar to a genuine one but not due to enzyme contained in the tissue, may be encountered occasionally. Only their causes will be enumerated here; their prevention will be dealt with under the individual methods.

1. The simplest mistakes result from confusing pigments with a positive reaction. Hemosiderin, for example, is converted by ammonium sulfide, a reagent often used in enzymatic histochemistry, into green-black ferrous sulfide. Although the shade of this compound is quite different from the gray-black of cobalt sulfide or from the brown-black of lead sulfide, obtained in the course of enzymatic reactions, errors from this source are not impossible.

2. Preformed calcifications in tissues are essentially similar in composition to some precipitates formed by enzyme activity and will be converted into the same colored end-products.

3. Heavy metals adsorbed by protein may constitute a source of error.

4. Certain substrates may undergo some degree of spontaneous hydrolysis or oxidation during incubation. This will result in false positive reactions by the secondary adsorption of reaction products.

5. Bacterial contamination of the substrate is a rare complication if proper precautions are taken (use of chloroform or camphor in the incubating medium), but it may occur once in a while, especially when incubation time is greatly prolonged. The medium may become grossly turbid, partly from the growth of microörganisms and partly from decomposition products of the substrate. The latter may be adsorbed on certain structures, causing false reactions.

On account of the possibility of false positives, it is necessary that, whenever a new method is proposed, the enzymatic nature of the reaction be proved. One or more of the

following control tests, run alongside the regular method, can be used to rule out nonenzymatic reactions:

1. Treat the sections prior to incubation with agents known to destroy enzymatic activity (boiling water, strong mineral acid, oxidants, heavy metals, etc.). It must be remarked that the resistance of enzymes varies widely; some are very easily destroyed, others are surprisingly resistant (e.g., hemoglobin is resistant to high temperatures and acids; myosin to precipitation by trichloroacetic acid). In some cases distinction between true and "pseudo"-enzymes must be arbitrary.
2. Use specific inhibitors (cyanide, azide, eserine, etc.) in an effective concentration.
3. Leave out the substrate from the incubating mixture.

Whatever reaction persists under the conditions of these control tests cannot be due to an enzymatic effect. Of course, the picture in the regular slide may reveal a combination of enzymatic and nonenzymatic reactions. In such a case only the difference between the regular and the control slide can be attributed to enzymatic activity.

The specificity of enzymes is rarely an absolute one. As a rule, one enzyme will attack several chemically related substrates (although the optimal conditions of activity may vary with the substrate). Conversely, one substrate may be attacked by a number of enzymes (although, again, optimal conditions may vary with the enzyme). Whether one or several enzymes are involved in any given reaction (or in related reactions) is a problem familiar to research workers in both bio- and histochemistry. In biochemistry the successful separation of a crude extract into fractions with distinctly different enzymatic properties is considered to be the evidence for the presence of several enzymes. In histochemistry, different topographical patterns of the distribution of activity, obtained under different conditions (varying the substrate, pH; the presence of activators or inhibitors) is assumed to be the indication for the involvement of more than

one enzyme. However, these different patterns must be quite constant and obtained under optimal conditions of precipitation in order to avoid the possible misinterpretation of random "all-or-none" effects and of diffusion artifacts.

The classification of histochemically demonstrable enzymes.—All enzymes for which histochemical techniques are known belong in one of the two groups: (1) oxidative, (2) hydrolytic enzymes.

1. OXIDATIVE ENZYMES

The oxidative enzymes fall into three groups: (*a*) dehydrogenases, (*b*) oxidases, and (*c*) peroxidases.

a) Dehydrogenases

The dehydrogenases catalyze the transfer of hydrogen to immediate acceptors other than oxygen and peroxides, although the ultimate acceptor may be oxygen. They are rather delicate enzymes which are largely destroyed by any sort of fixation and completely destroyed by embedding. They are rapidly inactivated even on standing. They require coenzymes, and some of them are also linked to the diaphorase or cytochrome systems.

The principle of their demonstration is the observation of the change in color of suitable hydrogen acceptors when they are reduced by the enzyme. The three main types of compounds used are: (1) methylene blue (Semenoff),[19] reduced to colorless leuco-methylene blue and thus indicating the sites of activity by bleaching; (2) various tetrazolium compounds, introduced into enzyme research by Kuhn and Jerchel,[20, 21] and Lakon;[22, 23] they are reduced to bright red,

19. Semenoff, W. E.: Ztschr. f. Zellforsch. u. mikr. Anat., **22**:305, 1934–35.

20. Kuhn, R., and Jerchel, D.: Ber. d. deutsch. chem. Gesellsch., **74**:941, 1941.

21. Kuhn, R., and Jerchel, D.: *ibid.*, **74**:949, 1941.

22. Lakon, G.: Ber. d. deutsch. bot. Gesellsch., **60**:299, 1942.

23. Lakon, G.: *ibid.*, **60**:434, 1942.

purplish, or blue formazans, insoluble in water and soluble in fats; and (3) tellurites (first introduced into bacteriological technique by Klett),[24] reduced to insoluble black elementary tellurium.

The methylene blue technique is not recommended because (*a*) the negative image it gives allows only a very poor localization and (*b*) the method is cumbersome and must be carried out under strictly anaerobic conditions to prevent the reoxidation of the leuco-base by atmospheric oxygen. The tetrazolium method is the most sensitive of the three and affords an excellent localization, except for an occasional secondary staining of fat droplets by formazan, a complication which should be borne in mind to avoid misinterpretation of the pictures. It should also be mentioned that some of the commercial batches of tetrazolium compounds may contain oxidizing substances (probably lead tetra-acetate) as an impurity and that these interfere with the reaction. The formation of dye should be quite noticeable after about 5–10 minutes of incubation if good active material is used (e.g., rat kidney); if not, the reagent must be recrystallized by dissolving it in a small volume of absolute alcohol and precipitating it with 4–5 volumes of ether. The tellurite method is considerably less sensitive, but it gives nice, sharp pictures.

Even without the use of any substrate, positive reactions will be obtained in most cases on account of the presence of various endogenous substrates in the tissues. Although such reactions of an undefinable substrate specificity are usually weak, the substrate being exhausted rapidly, one should try to avoid them by rinsing the sections before incubation. For all practical purposes, reactions obtained in rinsed sections will be specific for the dehydrogenases of the substrate supplied.

Maintaining the right pH under incubation is important. The optimum for the enzymatic activity is pH 7.3–7.6; at

24. Klett, A.: Ztschr. f. Hyg., **33**:137, 1900.

much higher pH values all the reagents mentioned will undergo nonenzymatic reduction by a number of substances, such as sulfhydryl compounds, polyphenols (adrenalin), etc.

Method

According to Seligman and Rutenburg[25] the tissue need not be absolutely fresh; refrigeration for 4 hours at 4° C. does not cause any noticeable loss of activity; in fact, even fixation in chilled acetone for 4 hours causes only 40 per cent inactivation of the enzyme.

Use frozen sections 25–50 μ thick. Thinner sections often fail to stain (destruction of cellular organization in the superficial layers or loss of cofactors by diffusion). Zweifach, Black, and Shorr[26] recommend the use of razor-blade hand sections, about 0.5–1 mm. thick. After the reaction has taken place, the pieces can be fixed in formalin, frozen, and cut. Loose cellular material (sediments, scrapings) are suspended in the incubating medium.

The composition of the substrate mixture is not critical, except for the pH (7.3–7.6). The concentration of the buffer (phosphate) should be 0.05–0.1 M; that of the substrate (succinate, lactate, etc.), 0.1–0.2 M. Triphenyltetrazolium chloride and neotetrazolium chloride[27] (a dimer of the former) are used in 0.5–1 per cent solutions; the more insoluble ditetrazolium chloride[28] (a dimethoxy derivative of neotetrazolium) and potassium tellurite (K_2TeO_3) in a 0.1 per cent solution. Incubation time at 37° C. will range from 20 minutes to 3 hours. The dye formed from triphenyltetrazolium is scarlet-red; that from neotetrazolium, purple-red; that from ditetrazolium, in thin layers purple, in thick layers deep blue. The slides should be examined within a few hours because for-

25. Seligman, A. M., and Rutenberg, A. M.: Science, **113**:317, 1951.

26. Zweifach, B. W., Black, M. M., and Shorr, E.: Proc. Soc. Exper. Biol. & Med., **74**:848, 1950.

27. Available from Pannone Chemical Co., Farmington, Conn.

28. Available from Dajac Laboratories, 511 Lancaster Ave., Leominster, Mass.

mazan dyes have a tendency to secondary organization into coarse crystalline precipitates. Elementary tellurium is black or brown-black. The sections can be counterstained with hematoxylin or carmine; they should be mounted in glycerol or glycerol-jelly.

b) Oxidases

Oxidases are a motley group of enzymes having in common the property of catalyzing the oxidation of various substrates, mainly phenols and amines, in the presence of atmospheric oxygen. Their classification is very unsatisfactory. Substrate specificity is usually only relative; the same enzyme will attack a number of substrates (although some of them more readily than others); and, conversely, the same substrate may be attacked by a number of different enzymes. It is impossible to go into the complicated and controversial details of the problem. The interested reader can seek more information in special textbooks of enzymology. Only a few enzymes of histochemical interest will be dealt with here.

A) *Indophenol oxidase* (*nadi oxidase; cytochrome oxidase*).—

A mixture of solutions of a phenol or naphthol and an aromatic diamine is slowly oxidized on exposure to air, with the formation of intensely colored (usually blue) indophenol dyes, most of which are insoluble in water but very soluble in oils and fats.

The reaction is immediate in the presence of strong oxidants, such as dichromate or hypochlorite. Ehrlich in 1885[29] showed by the injection of a mixture of alpha-naphthol and dimethyl-*p*-phenylene diamine into animals that the formation of indophenol blue is catalyzed by living tissues, in the absence of strong oxidants. Röhmann and Spitzer[30] found that ground-up tissues or even alcohol-ether-dried organ

29. Ehrlich, P.: Das Sauerstoffbedürfnis des Organismus (Berlin: A. Hirschwald, 1885).

30. Röhmann, F., and Spitzer, W.: Ber. d. deutsch. chem. Gesellsch., **28**:567, 1895.

powders had a strong catalytic effect. In 1907 Winkler[31] demonstrated that the same mixture, at an alkaline reaction, will stain pus cells in alcohol-fixed smears. Shortly afterward Schultze[32] adapted the stain for use on tissue sections and emphasized the diagnostic value of the method in distinguishing myeloid from lymphoid cells. From then on, for many years, the "indophenol oxidase" problem has been in the focus of interest. An extremely large number of papers has been published on its chemical background, its application, and the interpretation of its results. The term "nadi oxidase" (from a contraction of the first syllables of the two reagents), which has gained a wide acceptance, was coined by Graeff.[33]

In 1922 von Gierke[34] noticed that the distribution of the reaction is not the same in fresh tissues treated with an unalkalized reagent mixture as in fixed tissues treated with an alkalized one. Whereas in the first case the reaction is extremely widespread throughout all tissues and not particularly intense in myeloid elements, in the second case it is limited almost exclusively to the granules of myeloid leucocytes, which stain very intensely. Furthermore, the diffuse reaction (G for *Gewebe* = tissue nadi reaction of Graeff)[35] does not take place if freshly boiled distilled water is used as a solvent and is readily prevented by moderate heat (55° C.), by formalin, acetone, alcohol, acid, and alkali, etc. The myeloid reaction (M reaction of Graeff),[35] on the other hand, takes place even in oxygen-free water, is relatively heat-stable (up to 80° C.), insensitive to formalin (tissues kept in formalin for 6 years still give excellent reactions), to

31. Winkler, F.: Folia haemat., **4**:323, 1907.

32. Schultze, W. H.: Beitr. z. path. Anat. u. z. allg. Path., **45**:127, 1909; Schultze, W. H.: München. med. Wchnschr., **56**:167, 1909; Schultze, W. H.: *ibid.*, **57**:2171, 1910; Schultze, W. H.: Zentralbl. f. allg. Path. u. path. Anat., **28**:8, 1917.

33. Graeff, S.: Beitr. z. path. Anat. u. z. allg. Path., **70**:1, 1922.

34. Gierke, E. von: München. med. Wchnschr., **58**:2315, 1911.

35. Graeff, S.: Zentralbl. f. allg. Path. u. path. Anat., **32**:337, 1922.

acetone, alcohol, alkali (actually, the optimum pH of the reaction is 12.5–13), very little sensitive to dichromate and Lugol's solution and many other agents which will promptly destroy practically all the known enzymes. However, it is sensitive to acids.

In 1933 Keilin[36] came forward with the theory that the enzyme responsible for the G nadi reaction is identical with cytochrome oxidase. According to this theory, dimethyl-*p*-phenylene diamine is oxidized by cytochrome. Reduced cytochrome is then reoxidized by cytochrome oxidase in the presence of atmospheric oxygen. Keilin's ideas have become generally accepted, and the designation "cytochrome oxidase" has superseded the terms "labile" or "G" indophenol oxidase. Since cytochrome oxidase is a rather sensitive enzyme, readily destroyed even by drying and intolerant to formalin, etc., it is obvious that it cannot be responsible for the effects seen in the stable or M nadi reaction.

First of all, by definition the stable enzyme is not an oxidase, since it is effective in the absence of atmospheric oxygen. In fact, it is altogether questionable whether enzymatic action is involved in the stable reaction. It is difficult to conceive of an enzyme which is resistant to formalin indefinitely, to Lugol's solution, and to dichromate for over 30 minutes. The writer was unable to confirm the sensitivity of the reaction to $HgCl_2$, reported repeatedly. He could verify the findings of Heringa and Ruyter:[37] blood smears after 30 minutes' exposure to a 2 per cent solution of $HgCl_2$, followed by removal of the metal with Lugol's solution, were indistinguishable from those given by untreated smears. The exceedingly high pH optimum (around 13) is also an unlikely attribute of an enzyme. Graeff[33] and Katsunuma[38] voiced early opin-

36. Keilin, D.: Ergebn. d. Enzymforsch., **2**:239, 1933.

37. Heringa, G. C., and Ruyter, J. H. C.: Acta brev. Neerland., **5**:118, 1935.

38. Katsunuma, S.: Intrazelluläre Oxydation und Indophenolblausynthese (Jena: G. Fischer, 1924).

ions in favor of the catalytic but nonenzymatic nature of the reaction.

It is known that the formation of indophenol blue from the nadi reagent can be catalyzed nonenzymatically. The positive reaction given by unsaturated fats has been mentioned before (p. 110). Remesow[39] obtained positive reactions with irradiated cholesterol. In model experiments filter-paper strips moistened with a solution of linseed oil or oleic acid in chloroform were allowed to dry for 12–24 hours. An intense blue color was observed after 1 minute's immersion in the nadi reagent. Schümmelfeder[40] reported negative results with oleic acid in similar experiments. It must be assumed that he did not allow enough time for the formation of peroxides. The presence of peroxides in oleic acid was proved by Lison.[41] He shook a solution of benzidine and hemoglobin with oleic acid (in place of H_2O_2 as the peroxide) and obtained the typical shade of benzidine blue in the aqueous phase, just as if hydrogen peroxide had been employed. Nadi-positive lipid substances have been prepared by several workers.[42] The lipid nature of the granules of the myeloid elements has been demonstrated repeatedly, and the morphological appearance of the Sudan-stained substance is identical with that of the nadi-positive granules.[43] Sehrt[44] could prove that sudanophilia and staining by the nadi reaction invariably run parallel; if a lipid solvent abolishes one, it will abolish the other.

On the basis of these facts, it would seem that the enzymatic theory is a superfluous assumption; the formation of

39. Remesow, I.: Virchows Arch. f. path. Anat., **285**:591, 1932.

40. Schümmelfeder, N.: Virchows Arch. f. path. Anat., **317**:707, 1950.

41. Lison, L.: Bull. Soc. chim. biol., **18**:185, 1936.

42. Neumann, A.: Arch. f. exper. Zellforsch., **6**:298, 1928; Gutstein, M.: Biochem. Ztschr., **207**:177, 1929; Magat, J.: Compt. rend. Soc. de biol., **116**:1367, 1934.

43. Dietrich, A.: Ergebn. d. allg. Path. u. path. Anat., **13**:283, 1909; Marinesco, G.: Compt. rend. Soc. de biol., **82**:258, 1919.

44. Sehrt, E.: München. med. Wchnschr., **74**:139, 1927.

indophenol blue under the conditions of the nadi M reaction can be explained satisfactorily by the presence of fat peroxides in the myeloid granules. The simultaneous presence of aldehydes would greatly accelerate the reaction.[45] The inhibition by cyanide, often quoted as an evidence of the enzymatic nature of the reaction, actually does not prove anything. Cyanide is known to poison many inorganic catalysts; moreover, it may act simply as a powerful reducer. In the model experiments mentioned, cyanide completely inhibited the staining of the paper slips and very greatly slowed down the spontaneous oxidation of the nadi mixture in air.

This interpretation of the reaction renders the arguments meaningless as to whether the distribution of the reaction does or does not correspond to the true localization of the enzyme.[46]

One version of the enzymatic theory, however, cannot be refuted with certainty. Although the reaction will take place even in the absence of any enzyme, a uniquely hardy peroxidase, capable of utilizing fat peroxides, might be present and contribute its catalytic effect.

Methods

1) *For cytochrome oxidase (labile or G nadi reaction).*— Use small fragments of fresh tissue or razor-blade slices. Frozen sections of fresh unfixed tissue can also be used, although freezing may cause partial inactivation of the enzyme. To about 25 ml. of a phosphate buffer of pH 7.2–7.6 add 1–2 ml. of a 1 per cent solution of alpha-naphthol in 40 per cent alcohol and the same amount of a 1 per cent solution of dimethylparaphenylenediamine (*p*-aminodimethylanilin) hydrochloride. Incubate in a shallow dish for about 10 minutes or until the reaction is clearly visible. Rinse sec-

45. Feigl, F.: Qualitative analysis by spot tests (New York: Elsevier Publishing Co., 1946), p. 345.

46. Dietrich, A.: Zentralbl. f. allg. Path. u. path. Anat., **19**:3, 1908; Hollande, A. C.: Compt. rend. Acad. sc., **178**:1215, 1924; Prenant, M.: Bull. d'histol. appliq. à la physiol., **2**:329, 1925.

tions; if desired, counterstain with lithium carmine. Mount in glycerin-jelly. The preparations are not permanent. A positive reaction consists of a blue color.

2) *For fat peroxides.* (*a*) *Stable or M nadi reaction.*—Fix in formalin-alcohol or formalin. Frozen sections are preferable, but excellent results are often obtained with paraffin sections. Smears should be fixed in 95 per cent alcohol or formalin-alcohol (1:9). The incubating mixture is similar to the previous one, except for the pH. Instead of phosphate buffer, use distilled water, and add 2–3 drops of strong ammonia water or N NaOH to 50 ml. of the mixture. A positive reaction consists of a blue color.

The results of this method can be checked by the (*b*) *Glavind-Granados-Hartmann-Dam technique.*[47] Prepare two solutions: A, dissolve 40 mg. hematin in 10 ml. of pyridine, add 20 ml. of glacial acetic acid; B, dissolve 25 mg. of leuco-2,6-dichlorophenolindophenol in 3.5 ml. of absolute alcohol, add 5 ml. distilled water. This latter solution is unstable and must be used fresh. Mix 0.75 ml. of solution A with the total of solution B and apply the mixture to the sections (or smears) for 3–5 minutes. Wash in water, mount in glycerol-jelly. Fatty peroxides stain red.

B) *Nonspecific phenol oxidase.*—

Dark brownish or purplish staining of the granules of myeloid leucocytes can be obtained by incubating alcohol- or formalin-alcohol-fixed smears with slightly alkaline solutions of various phenols, such as tyrosin, adrenalin, hydroquinone,[48] alpha-naphthol,[49–51] and dioxyphenylalanin.[52] The conditions of this reaction have not been investigated in de-

47. Glavind, I., Granados, H., Hartmann, S., and Dam, H.: Experientia, **5**:84, 1949.

48. Kreibich, C.: Wien. klin. Wchnschr., **23**:701, 1910; Kreibich, C.: *ibid.*, **23**:1443, 1910.

49. Loele, W.: München. med. Wchnschr., **57**:1394, 1910.

50. Loele, W.: Die Phenolreaktion (Aldaminreaktion) und ihre Bedeutung für die Biologie (Leipzig: W. Klinkhardt, 1920).

51. Loele, W.: Virchows Arch. f. path. Anat., **262**:39, 1926.

52. Bloch, B., and Peck, S. M.: Folia haemat., **41**:166, 1930.

tail, and no opinion can be expressed as to whether or not it is of an enzymatic nature.

It must be remarked here that myeloid granules show peculiar reactions when incubated with phenolic substances (especially naphthols) under the conditions mentioned. If the solutions used are stale and somewhat colored by spontaneous oxidation, an intense purple-black coloration of the granules becomes evident within 10 minutes. With fresh solutions, darkening of the granules is absent or minimal; however, the binding of naphthol by the granules can easily be demonstrated by suitable reactions, such as azo-coupling or the Gibbs reaction (formation of indophenol blue on exposure to dichloro- or dibromoquinoneimide). Moreover, the naphthol-treated granules exhibit unusual staining reactions. They will stain intensely with basic dyes[50, 53] and with fat dyes, such as Sudan III (de Bruijn).[54] Even in paraffin sections, myeloid granules will stain intensely with Sudan III or Sudan Black B after 10 minutes' exposure to an alkalized, saturated aqueous solution of naphthol. The nature of this "phenolophilia" or "naphtholophilia"[50] is not clear (quinone-ethylenic condensation?).

C) *Dopa oxidase.*—

In 1916 Bloch and Ryhiner[55] found that the basal layers of the epidermis, leucocytes, and other tissue elements contain an enzyme which oxidizes *l*-3,4-dihydroxyphenylalanin (dopa) to a blackish pigment, indistinguishable from natural melanin. In subsequent papers[56] Bloch offered good evidence that dopa is attacked by two different enzymes: (1) a non-specific phenol oxidase which is relatively resistant to various

53. Graham, G. S.: J. M. Research, **35**:231, 1916–17.

54. De Bruijn, P. P. H.: Acta Neerland. morph. norm. et path., **2**:232, 1939.

55. Bloch, B., and Ryhiner, P.: Ztschr. f. d. ges. exper. Med., **5**:179, 1916–17.

56. Bloch, B., and Löffler, W.: Deutsches Arch. f. klin. Med., **121**:262, 1916–17; Bloch, B.: Ztschr. f. physiol. Chem., **98**:226, 1917; Bloch, B.: Arch. f. Dermat. u. Syph., **124**:129, 1917; Bloch B.: Am. J. M. Sc., **177**:609, 1929; Bloch, B., and Schaaf, F.: Klin. Wchnschr., **11**:10, 1932.

chemical and physical agents and is present in a number of structures, especially in myeloid leucocytes, and (2) an absolutely specific dopa oxidase which does not act on any other substrate, is easily inactivated by chemical and physical agents, and is present only in cells concerned with the elaboration of melanin (basal layer of epidermis, chromatophores, cells of melanoma, etc.). Although Bloch's theory was criticized by several authors (Heudorfer,[57] Przibram[58]), it appears that the virtual specificity of the enzyme of melanin-producing cells in vertebrate species is a well-established fact. The only compound, besides dopa, to be attacked by this enzyme is oxytyramin (Mulzer and Schmalfuss).[59] In insects and other species, other melanizing enzyme systems may be present (Hasebroek),[60] and even vertebrates possess other enzymes which will convert tyrosin and related compounds to melanin. The pathway of melanin formation by both types of enzymes is essentially the same and leads through derivatives of indole[61] (ring-closure of the side chain). To rule out nonspecific phenol oxidases, it is advisable to incubate control sections with a different substrate (e.g., naphthol or tyrosin).

Method (Laidlaw and Blackberg's[62] modification)

Use frozen sections of fresh material or of tissue fixed for only a few hours in 5 per cent formalin. Longer fixation may cause partial inactivation of the enzyme. Rinse sections very briefly in distilled water and transfer them into a 0.1 per cent solution of dihydroxyphenylalanin (the commercial substance is a mixture of the stereo-isomers), buffered with a phosphate buffer to pH 7.3–7.5, in an open dish for 4–5

57. Heudorfer, K.: München. med. Wchnschr., **68**:266, 1921; Heudorfer, K.: Arch. f. Dermat. u. Syph., **134**:339, 1921.
58. Przibram, H.: Arch. f. Entwcklngsmech. d. Organ., **48**:140, 1921.
59. Mulzer, P., and Schmalfuss, H.: Med. Klin., **27**:1099, 1931.
60. Hasebroek, K.: Fermentforsch., **5**:1, 1922.
61. Dulière, W. L., and Raper, H. S.: Biochem. J., **24**:239, 1930; Mason, H. S.: J. Biol. Chem., **168**:433, 1947.
62. Laidlaw, G. F., and Blackberg, S. N.: Am. J. Path., **8**:491, 1932.

hours. Temperature should be between 20° and 37° C. It is advisable to change the incubating solution once or twice to avoid the deposition of a melanin precipitate (by spontaneous oxidation of the substrate). At pH 7.7 the reaction is much faster (about 1 hour), but the danger of precipitates is also increased. Rinse sections, counterstain as desired, dehydrate, and mount. Sites of dopa oxidase activity appear dark brown-gray or brown-black. For greater contrast, melanin formed during the reaction can be blackened by silver (see under "Pigments").

D) *Amine oxidase.*—

This enzyme, originally described by Hare,[63] oxidizes aliphatic and aromatic amines, such as isoamylamine, tyramine, histamine, tryptamine, etc., first to the corresponding aldehydes and then to acids. Ammonia and hydrogen peroxide are formed in the course of the reaction. The corresponding amino acids (tyrosin, histidine, etc.) are not attacked.

Oster and Schlossmann[64] described a method for the histochemical localization of amine oxidase, based on the demonstration of the aldehyde formed. The detailed technique is as follows:

Fresh-frozen sections are used. Since tissues contain plasmalogen, which, on standing, may break down to plasmal, an aldehydic compound, it is necessary first to transform plasmal into a nonreacting form to avoid its being mistaken for newly formed aldehyde. This is accomplished by treating the sections with a solution of bisulfite; the bisulfite addition compound of plasmal does not react with the usual aldehyde reagents. After thorough washing, the sections are incubated with the substrate (tyramine) and treated with Schiff's reagent. The aldehyde formed is demonstrated by the localized development of an indigo-blue shade.

The theoretical foundation of this method is not sound.

63. Hare, M. L. C.: Biochem. J., **22**:968, 1928.

64. Oster, K. A., and Schlossman, N. C.: J. Cell. & Comp. Physiol., **20**:373, 1942.

First of all, neither the enzyme[65] nor the aldehyde formed (*p*-hydroxyphenylacetaldehyde) is insoluble enough to remain at its original site; both would diffuse out into the solution. Second, the oxidation of tyramine by amine oxidase goes beyond the aldehyde stage[63, 66] unless the aldehyde formed is promptly trapped (e.g., by semicarbazide).

In addition to the theoretical objections, it can be shown experimentally that positive reactions are obtained even in the absence of substrate and after the treatment of the sections with chemicals which almost certainly destroy the enzyme (trichloroacetic acid).[67]

The chemical nature of the aldehyde giving the peculiar blue shade with Schiff's reagent is not known, but it appears to be either a modified form of plasmal or some other lipid aldehyde produced from unsaturated fats by nonenzymatic oxidation. It is not likely to be an aromatic compound, such as would be formed by enzymatic action, because the shades given by simple aromatic aldehydes are much more reddish.

c) Peroxidases

Enzymes of the peroxidase group catalyze the transfer of oxygen from hydrogen peroxide and other peroxides to a variety of acceptors. Chemically, most if not all of the peroxidases appear to be heme proteins. They are quite resistant to various chemical and physical agents, especially to acids and heat.

It must be remembered that some substrates may be attacked by peroxides (not necessarily all peroxides) directly[68] and that some of the catalysts are not of protein nature (iron porphyrins; in some instances even inorganic Fe salts in conjunction with ascorbic acid).[69] Therefore, a reaction obtained

65. Blaschko, H., Richter, D., and Schlossmann, H.: Biochem. J., **31**:2187, 1937.

66. Bernheim, M. L. C.: J. Biol. Chem., **93**:299, 1931.

67. Gomori, G.: Ann. New York Acad. Sc., **50**:968, 1950.

68. Dixon, M.: Biochem. J., **28**:2061, 1934.

69. Bezssonoff, N., and Leroux, H.: Bull. Soc. chim. biol., **28**:286, 294, 608, 1946.

with peroxide and a suitable substrate is not, in itself, a cogent proof for the presence of true peroxidase. Even when it is known that a morphological structure does contain peroxidase (e.g., leucocytes), it is always questionable whether the reaction obtained under the conditions of the histochemical experiment is due to enzymatic action or to a nonprotein catalyst ("pseudo-peroxidase").

For the histochemical demonstration of peroxidase, benzidine,[70] naphthol,[71] and various leuco-dyes are utilized[72, 73] in the presence of hydrogen peroxide. Benzidine is oxidized to a blue (quinhydrone) or brown (quinoneimine) dye; naphthol to a purple-black one, the chemical nature of which is not clear; leuco-dyes are recolorized to their original shades. It is important to run controls without peroxide because positive reactions may be obtained even in its absence (see previous section, "Phenol Oxidase").

The two main histological localizations of peroxidase are hemoglobin and myeloid granules. However, the reactions given by hemoglobin differ from those obtained in myeloid granules in several important points:

1. Zinc leuco-dyes are readily recolorized by hemoglobin but not by myeloid granules.

2. In the benzidine reaction the optimal concentration of H_2O_2 is about 0.01 M in the case of myeloid granules and much higher, about 0.1 M, in the case of hemoglobin.

3. The activity of myeloid granules is rapidly destroyed by heating to 75°–80° C. or by extraction with a warm chloroform–methyl alcohol mixture. The activity of hemoglobin is entirely resistant to these influences.

In model experiments, stale (peroxidized) linseed oil imbibed in filterpaper strips gives rather intense reactions with benzidine but none with leuco-dyes or with naphthol. An-

70. Adler, O., and Adler, R.: Ztschr. f. physiol. Chem., **41**:59, 1904; Lepehne, G.: Beitr. z. path. Anat. u. z. allg. Path., **65**:163, 1919; Goodpasture, E. W.: J. Lab. & Clin. Med., **4**:442, 1919.

71. Loele, W.: Folia haemat., **14**:26, 1912.

72. Lison, L.: Compt. rend. Soc. de biol., **106**:1266, 1931.

73. Jacoby, F.: J. Physiol., **103**:25P, 1944–45.

other interesting observation is the fairly intense staining of both myeloid granules and cell nuclei with peroxide-free acidified solutions of benzidine, prepared with tap water (effect of free chlorine?).

These facts clearly indicate that the oxidation of various substrates by peroxides can be promoted by more than one mechanism; in fact, even the invariable involvement of peroxides in what is called a "peroxidase reaction" is not certain. The clarification of this problem will require much more investigative work.

In histochemistry the most important application of the peroxidase reaction is the demonstration of hemoglobin. After hemolytic reactions, hemoglobin retains its enzymatic properties (in renal tubules) for only 24–36 hours, although its other staining reactions remain unchanged for several days.

Methods

Smears are fixed with acetone, alcohol, or formalin-alcohol (1:10). For tissues the same fixatives or formalin-saline are recommended. It is important that the fixative should not hemolyze the red cells, thereby causing a diffusion of the reaction for hemoglobin. Frozen sections are the best, although in most cases excellent results are obtained after celloidin- or paraffin-embedding.

A) *Lison's*[72] *zinc-leuco method* gives the best results for fresh hemoglobin. Prepare the following staining solution: Dissolve 1 g. of either acid fuchsin, acid violet, or patent blue in 100 ml. of a 2 per cent acetic acid solution; add 5–10 g. of zinc dust, and boil mixture until the dye is decolorized (patent blue will bleach only to a pale brown). Add 2 more ml. of concentrated acetic acid. Keep the mixture in the icebox. In the case of recolorization, heat briefly until decolorized once more. For use, filter 10 ml. and add 1 ml. of commercial (3 per cent) hydrogen peroxide to it. Pour mixture over the slide. Hemoglobin will stain very intensely almost

immediately, in the shade of the original dye. Counterstain as desired. Mount in balsam.

B) *The benzidine method* will stain even somewhat stale, degraded hemoglobin (nonenzymatic heme catalysis?). Of the numerous modifications of this method, the technique of Osgood[74] and Washburn[75] is recommended.

Prepare a 0.2–0.3 per cent solution of benzidine in 95 per cent alcohol. To each 100 ml. of the solution add about 0.5 g. Na nitroprusside dissolved in a few ml. of water (nitroprusside appears to enhance blue shades in preference to brown ones). This stock solution will keep for months if refrigerated.

When staining smears, pour the solution over the slide for 3 minutes. The alcoholic solution will serve as a fixative. This step may be omitted in the case of tissue sections. Decant. Mix equal volumes of the solution and a 1:5 dilution (for hemoglobin) or a 1:50 dilution (for myeloid granules) of commercial (3 per cent) hydrogen peroxide; pour it over the slide and leave it on for about 5 minutes. Decant, wash slide briefly in water, counterstain with a red nuclear stain, dehydrate, and mount in balsam. Myeloid granules appear intensely dark blue; hemoglobin appears in shades of dark brown to blue. The addition of a few drops of an acetate buffer of about pH 4.5 to the benzidine-H_2O_2 mixture will make hemoglobin stain in a clearer blue shade. The naphthol technique of Loele[71] is not recommended.

APPENDIX

Unna's "Reduktionsorte" and "Sauerstofforte"

The terms *Reduktionsorte* ("sites of reduction") and *Sauerstofforte* ("sites of oxygen") were coined by the German dermatologist Unna[76] to denote structures which reduce oxidizing substances or show the presence of "free" oxygen, respectively. The first are demonstrated by

74. Osgood, E. E.: Atlas of hematology (San Francisco: Stacey, 1937).

75. Washburn, A. H.: J. Lab. & Clin. Med., **14**:246, 1928.

76. Unna, P. G.: Arch. f. mikr. Anat., **78**:1, 1911; Unna, P. G.: Med. Klin., **8**:951, 1912; Unna, P. G.: Berl. klin. Wchnschr., **50**:809, 1913; Unna, P. G.: Abderhalden's Handb. d. biol. Arbeitsmeth., **V-2²**:62, 1928.

potassium permanganate (brown coloration) or by ferriferricyanide (precipitation of Prussian blue), the latter by the recolorization of leuco methylene blue.

The application of the modern redox-indicator dyes to the tissues[77] should give results far more delicately graded than the methods of Unna. However, it must be admitted that the oxidizability of organic compounds by permanganate or by ferricyanide is theoretically meaningful: it indicates the presence, in the structures stained, of compounds with oxidation-reduction potentials lower than those of the oxidants mentioned. Owing to its very powerful oxidizing properties, the specificity of permanganate is practically nil, since it is reduced by the majority of tissue components. Ferricyanide, being a much milder oxidant, does give a fairly selective staining of the more strongly reducing groups (–SH, polyphenols). On the other hand, the results of staining with leuco methylene blue cannot by any means be construed to reveal anything about the distribution of "free" oxygen (whatever this term may mean) or of oxidizing systems in the tissues. Unna stained sections in a solution of leuco methylene blue containing a large excess of the reducing agent (formaldehyde sulfoxylate). While the tissue is in this solution, it shows no indication of staining. On rinsing the sections in water, the color of methylene blue becomes apparent, and the picture is very similar to, although not absolutely identical with, that obtained with a regular solution of methylene blue. Obviously, the affinity of leuco-dye to basophilic structures is practically the same as that of the parent-dye, allowing for minor differences caused by structural changes in the dye molecule. When the excess of the reducer is removed by rinsing and the tissue is exposed to atmospheric oxygen, the latter will restore the bound leuco-dye to its original color. There is absolutely no need to assume any oxidizing activity residing in the tissue proper. Unna's work has been received wtih much criticism[78] but has been defended stubbornly by the author, who ultimately resorted to such fanciful hypotheses as the "oxypolar affinity,"[79] meaning some sort of chemical attraction between compounds rich in oxygen and poor in oxygen.

A slight modification of Unna's leuco methylene blue technique was revived by Roskin.[80] as a diagnostic aid for the recognition of malig-

77. De Robertis, E., and Moura Gonçalves, J.: Endocrinology, **36**:245, 1945.

78. Oelze, F. W.: Arch. f. mikr. Anat., **84**:91, 1914; Schneider, H.: Ztschr. f. wissensch. Mikr., **31**:51, 478, 1914; Rothman, S.: Jadassohn's Handb. d. Haut- u. Geschlechtskrankh., I, **2**:330, 1929.

79. Unna, P. G.: Arch. f. mikr. Anat., **87**:96, 1915.

80. Roskin, G.: Ztschr. f. Krebsforsch., **35**:140, 1931; Roskin, G.: Ztschr. f. Zellforsch. u. mikr. Anat., **14**:781, 1931–32; Roskin, G., and Semenoff, W.:

nant changes. Roskin and co-workers and Voinov[81] assert that malignant cells (in unfixed smears) do not stain with the dye while nonmalignant ones do. Ludford[82] and the present writer[83] were unable to verify Roskin's claims.

2. HYDROLYTIC ENZYMES

The hydrolytic enzymes demonstrable histochemically belong, with the exception of two individual enzymes—phosphamidase and glucuronidase—in the group of esterases; that is, they hydrolyze esteric linkages. In modern usage the term "esterase" is restricted to those enzymes with a substrate specificity for carboxylic esters. The other ester-splitting enzymes derive their names from the acid components of the esters they hydrolyze preferentially, since it appears that their specificity is determined by the acid moiety (phosphatase, sulfatase).

Methods for Hydrolytic Enzymes in General

Depending on the substrate, hydrolysis yields an acid ion and an alcohol or a phenol (or, in the case of phosphamidase, an acid and an amide). Reactions have been devised for the demonstration of either the acid or the alcoholic moiety.

The *acids* are demonstrated by their regular precipitation reactions with metal ions. Theoretically, many cations could be used to trap the acid ions; in practice, however, only a few are suited for histochemical application, the majority being too toxic to enzymes or incompatible with the substrate at the optimal pH of the reaction. The ones most often used are calcium and lead; cobalt, iron, and copper are employed in one method each.

The principle is to add a cation, known from analytical

Ztschr. f. Zellforsch. u. mikr. Anat., **19**:150, 1933; Roskin, G., and Solowjewa, W.: Acta cancrol., **1**:464, 1934–35; Roskin, G.: Bull. biol. et méd. expér., **3**:375, 1937; Roskin, G.: Bull. d'histol. appliq. à la physiol., **15**:20, 1938; Roskin, G., and Struve, M. E.: Stain Technol., **22**:83, 1947.

81. Voinov, V. A.: Klinicheskaia meditsina, **18**:51, 1940.
82. Ludford, R. J.: Arch. f. exper. Zellforsch., **17**:339, 1935.
83. Gomori, G.: Unpublished.

chemistry to form a highly insoluble precipitate with the acid ion in question, to the incubating mixture. As the acid is liberated, its ions will be trapped *in statu nascendi* by the cation, to yield a precipitate at the site of formation.

The precipitate formed is usually colorless and not easily seen under the microscope. Therefore, it must be transformed into a colored, easily observable compound. In the case of the heavier metals the sections can be treated directly with a suitable reagent. Soluble sulfides, for example, will transform precipitates of Pb, Co, Fe, and Cu into blackish, exceedingly insoluble sulfides. Fe and Cu can also be transformed into the corresponding ferrocyanides (blue and red-brown, respectively). When the primary precipitate is a Ca salt, it can be demonstrated by the methods mentioned in the section on calcium (p. 33).

The *alcoholic* (or phenolic) moiety can be demonstrated only if it is a thio-alcohol or a naphthol. Some thio-alcohols form highly insoluble precipitates with heavy metals; naphthols can be visualized as azo dyes.

The azo-coupling reaction is rapidly becoming the basis of several excellent histochemical techniques and therefore deserves a somewhat detailed discussion. Its application to insoluble tissue phenols has been described in the section on phenolic substances (p. 120). In the case of more soluble substances, such as are produced by enzymatic hydrolysis, a number of other important points have to be considered.

Under suitable conditions, diazonium salts will couple with aromatic amines and hydroxy compounds (and, in addition, with a number of heterocyclic compounds) to form brightly colored, very insoluble azo dyes. Hydroxides (phenols and naphthols) couple optimally at an alkaline reaction (pH 8 and up), whereas amines couple at an acid reaction (pH 3–5). This is only a general rule; while it is true that the coupling ability of phenols and naphthols rapidly declines as the neutral point is approached, there are a few types of naphthols which couple quite readily, almost at the

rate of an ionic reaction, even at pH 6.3–6.5. Amines (naphthylamines) couple much more slowly, and the sluggishness of the reaction precludes the use of azo-coupling for their histochemical demonstration (diffusion artifacts).

Any naphthol will not couple indiscriminately with any diazonium compound. Each new combination must be tried out in the test tube before its application to histochemistry can be considered. If coupling does take place, the color of the azo dye formed depends on both the naphtholic and the diazoic components used. The shade produced by any specific combination is not necessarily uniform but depends to some extent on factors such as the concentration of the ingredients, pH, temperature, etc.

Azo dyes of phenols are not sufficiently insoluble for histochemical use; for this reason, only naphtholic compounds are employed. Azo dyes formed from β-naphthol have very brilliant shades but have a tendency to remain in a colloidal solution for a short time before precipitating. This will cause some loss of fine structural details and false localizations (staining of acidophilic structures) around centers of high activity.[1] Even the addition of high concentrations of salt to the incubating mixture (making the dye more insoluble by the "salting-out" effect) does not help much. There is also a possibility that the coupling process itself is a relatively slow one in the case of β-naphthol, especially at pH values lower than about 8.5. On the other hand, α-naphthol and especially certain derivatives of β-naphthol, e.g., the naphthols of the AS series (chemically, substituted amides of 2-hydroxy-3-naphthoic acid), give exceedingly sharp and detailed pictures. The shades given by α-naphthol are somewhat dull; those given by the AS naphthols, very brilliant.

Until recently, the only naphtholic substrates readily available on the market were the acetate of α-naphthol and the benzoate of β-naphthol. At present, practically all the important substrates are made commercially. The Na salts of

1. Gomori, G.: J. Lab. & Clin. Med., **35**:802, 1950.

the phosphates and sulfates are quite soluble in water; esters of organic acids are very poorly soluble and require the addition of some acetone or propylene glycol to the incubating mixture to obtain clear solutions. Fortunately, the substrate concentrations required are very low (3–10 mg. of crystalline substrate to a Coplin jar, or 0.00015–0.0005 M).

Diazonium salts, which formerly had to be synthesized freshly for each experiment, are now available commercially in a wide variety and in a stabilized form. As dry powders, they can be kept in the icebox, if protected from light and moisture, almost indefinitely. However, their solutions are quite unstable and should be used immediately. Some of them keep better than others, but, on standing, even the most stable will decompose within about 2 hours. The rate of decomposition increases steeply with temperature and pH. The decomposition products are dark and have a tendency to stain the background, especially acidophilic structures, in a murky brownish shade. If incubation must be extended beyond 15–20 minutes, it is advisable to cool the substrate mixture with ice and to employ the lowest possible pH value compatible with good enzymatic activity and prompt azo-coupling. If longer incubation is carried out, the entire mixture should be renewed every 30 minutes or so.

Table 1 lists diazonium compounds which have been found to be useful because they give intense shades and are relatively stable.

The azo dyes formed from tetrazotized dianisidine and α-naphthol are insoluble in alcohol or xylol (but soluble in a mixture of the two; blot slide carefully between the last alcohol and xylene) and can be mounted in balsam. All other azo dyes formed from the ingredients mentioned are more or less soluble in absolute alcohol and especially in xylene. They must be mounted in glycerol-jelly or some similar medium.

As mentioned, azo-coupling either does not take place at all or is sluggish below pH 6. The pictures obtained, even in the best case, lack precision of detail, and centers of activity

are surrounded by a colored halo. To circumvent this difficulty, Seligman and associates[2] have suggested the use of esters of highly insoluble naphthols (such as 6-bromo-β-naphthol), in the absence of a diazonium salt, whenever incubation must be carried out at a low pH. The precipitated naphthol could then be azo-coupled in a second step, at an alkaline reaction. The idea sounds feasible but does not work well in practice. Naphthols insoluble enough to precipitate with a sharp localization will not couple unless enough organic solvent (acetone, alcohol) or strong alkali is added to effect some degree of solution. However, under such conditions, gross blurring of the picture will result. For this rea-

TABLE 1

Composition and Trade-Name of Compound*	To Be Used with Naphthol	Shade of Azo Dye
Tetrazotized *o*-dianisidine (Diazo Blue B Salt)	α	Purple-black
	β	Purple
	AS	Deep blue
Diazotized naphthylamine	α	Black
	β	Carmine red
	AS	Carmine red
Diazotized 4-chloro-2-aminoanisole (Diazo Red RC Salt)	α	Red-brown
Diazotized 3-nitro-4-aminoanisole (Diazo Bordeaux GP Salt)	α	Purple-brown
Diazotized 5-nitro-2-aminoanisole (Diazo Red B Salt)	α	Red-brown
Diazotized 3-nitro-4-aminoanisole (Diazo Red G Salt)	α	Red-brown
Diazotized 4-nitro-2-aminoanisole (Diazo Scarlet R Salt)	α	Red-brown
Diazotized ortho-amino-azotoluene (Diazo Garnet GBC Salt)	β	Red
	AS	Carmine red
Diazotized 4-benzoylamino-2,5-dimethoxy-aniline (Diazo Fast Blue RR Salt)	α	Black
	β	Purplish red
	AS	Purplish blue

* The proprietary names of these compounds may vary slightly with individual manufacturers. However, if ordered by the trade-names listed above, they will be readily identified.

2. Seligman, A. M., Nachlas, M. M., Manheimer, L. H., Friedman, O. M., and Wolf, G.: Ann. Surg., **130**:333, 1949.

son, the azo dye technique is not recommended for the demonstration of enzymes which require incubation at a pH lower than 6.3.

Esters of α- and β-naphthol are usually so rapidly hydrolyzed that diffusion artifacts result unless the incubating mixture is agitated all the time (p. 146). The simplest way of insuring thorough agitation is to use a low-speed electric or air-pressure stirrer with a long, narrow (about ½ inch wide) strip of stainless steel as a paddle, immersed almost to the bottom of the Coplin jar. Only two slides can be stained at one time; one in the first and one in the fifth slot of the Coplin jar; the tissues should face the stirrer. With esters of naphthol-AS which are hydrolyzed relatively slowly, stirring is not necessary.

Phosphatases

The enzymatic hydrolysis of phosphoric esters by animal tissues was first described by Grosser and Husler[3] in 1912. Ever since this original publication, the phosphatases have attracted a tremendous amount of scientific interest, and their literature has grown so vast that its mere review would fill a sizable volume.

The classification of the phosphatases is not entirely satisfactory and is based partly on substrate specificity and partly on pH optima. A large number of individual phosphatases have been described; in some instances, however, the criteria of specificity are not sufficiently clear-cut.

It should be remarked that, by a somewhat loose usage of terms, the existence of specific enzymes may be implied without any proof. For instance, the hydrolysis of glycerophosphate in the neutral range has been reported under the title of "neutral glycerophosphatase," although, in all likelihood, it is due to the combined activities of nonspecific acid and alkaline phosphatases, measured at a nonoptimal pH. Even if such a possibility is admitted later in the text, it would be more correct to avoid the use of such ambiguous terms. There

3. Grosser, P., and Husler, J.: Biochem. Ztschr., **39**:1, 1912.

is some overlapping between individual enzymes and groups of enzymes; thus alkaline phosphomonoesterase will hydrolyze nucleic acids quite readily, although most of the phosphate is in diesteric linkage; some pyrophosphatases will attack adenosinetriphosphate.

Table 2 is essentially the classification of Folley and Kay,[4] expanded somewhat to include more recent data. It contains most but not all of the dephosphorylating enzymes whose specificities are reasonably well established. For more detailed information, the reader is referred to textbooks of enzymology.[5]

Of the large variety of phosphatases, histochemical methods are available for nonspecific alkaline phosphatase, for 5-nucleotidase, acid phosphatase, and phosphamidase. A few data have been published on pyrophosphatase[6, 7] and metaphosphatase.[6, 7] Glick and Fischer[8] reported the histochemical localization of adenosinetriphosphatase in plant and animal tissue; but the specificity of their results has been questioned.[9] Soulairac and Desclaux[10] obtained positive results for adenosinetriphosphatase in rat muscle. Landschütz[11] found that adenosinetriphosphatase can be demonstrated in the cells of the Ehrlich mouse carcinoma.

No histochemical methods have been devised for the remainder of the group. Some of the phosphatases are so sensitive that they will not tolerate fixation and/or embedding (hexosediphosphatase, adenosinetriphosphatase of muscle).

4. Folley, S. J., and Kay, H. D.: Ergebn. d. Enzymforsch., **5**:159, 1936.

5. Albers, H.: Phosphatasen. In Nord and Weidenhagen's Handb. d. Enzymologie (Leipzig: Akad. Verlagsgesellschaft., 1940), **1**:408; Roche, J.: Phosphatases. In Sumner and Myrbäck's The enzymes (New York: Academic Press, 1950), I, **1**:473.

6. Nickerson, W. J., Krugelis, E. J., and Andresen, N.: Nature, **162**:192, 1948.

7. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **70**:7, 1949.

8. Glick, D., and Fischer, E. E.: Science, **102**:429, 1945.

9. Moog, F., and Steinbach, H. B.: Science, **103**:144, 1946.

10. Soulairac, A., and Desclaux, P.: Compt. rend. Soc. de biol., **143**:470, 1949.

11. Landschütz, C.: Experientia, **6**:232, 1950.

TABLE 2

CLASSIFICATION OF PHOSPHATASES

Name of Enzyme and Site of Occurrence	pH Optimum	Substrate
I. Phosphomonoesterases:		
a) Acid (Taka,[a] yeast,[b] liver[c])	3–4	Glycerophosphate
b) Acid (Spleen,[d] prostate,[e] citrus fruit[f])	4.5–5.5	Various monoesters
c) Neutral		
1. Yeast,[g] red cells[h]	Around 6	Various monoesters
2. Glucose-6-phosphatase[i] (liver)	6.5	Glucose-6-phosphate
d) Alkaline		
1. Nonspecific (various organs)	Around 9	Various monoesters, nucleic acid
2. Hexosediphosphatase (liver)[j]	Around 9	Hexosediphosphate
3. 5-nucleotidase[k] (nervous tissue, muscle)	Around 8	5-nucleotides
II. Phosphodiesterases:		
Bran,[l] snake venom,[m] serum[n]	4.5, 6.8, and 8.5	Diesters (diphenylphosphate)
III. Pyrophosphatase[o]	3.5–8.9	Salts and esters of pyrophosphoric acid
IV. Metaphosphatase[p]	7	Salts of metaphosphoric acid
V. Unclassified special phosphatases:		
a) Adenosinetriphosphatase		
1) Muscle[q]	7.6	Adenosinetriphosphate
2) Snake venom[r]	8.4	Adenosinetriphosphate
b) Phytase[s]	5.5 and 7.8	Phytin
c) Lecithinase[t]	5.1–7.6	Lecithin
d) Phosphoprotein phosphatase[u]	5–6	Casein
e) Polyphosphatase[v]	7	Salts of polyphosphoric acids
VI. Phosphamidase[w] (various organs)	5.6–6 and 9	Substituted amides of phosphoric acid

[a] Inouye, K.: J. Biochem. (Japan), 10:133, 395, 1928.
[b] Albers, H., and Albers, E.: Ztschr. f. physiol. Chem., 235:47, 1935.
[c] Bamann, E., and Salzer, W.: Biochem. Ztschr., 286:147, 1936.
[d] Davies, D. R.: Biochem. J., 28:529, 1934.
[e] Kutscher, W., and Wolbergs, H.: Ztschr. f. physiol. Chem., 236:237, 1935.
[f] Axelrod, B.: J. Biol. Chem., 167:57, 1947.
[g] Schäffner, A., and Bauer, E.: Ztschr. f. physiol. Chem., 232:66, 1935.
[h] Roche, J.: Biochem. J., 25:1724, 1931; Roche, J., and Bullinger, E.: Enzymologia, 7:278, 1939.
[i] Swanson, M. A.: J. Biol. Chem., 184:647, 1950.
[j] Gomori, G.: J. Biol. Chem., 184:647, 1950; Roche, J., and Bouchilloux, S.: Bull. Soc. chim. biol., 32:739, 1950.
[k] Reis, J.: Bull. Soc. chim. biol., 16:385, 1934; Reis, J.: Enzymologia, 2:110, 1937–38; Gulland, J. M., and Jackson, E. M.: Biochem. J., 32:597, 1938.
[l] Imanishi, Y.: Biochem. Ztschr., 237:406, 1932; Uzawa, S.: J. Biochem. (Japan), 15:11, 1932.
[m] Uzawa, S.: J. Biochem. (Japan), 15:19, 1932; Gulland, J. M., and Jackson, E. M.: Biochem. J., 32:590, 1938.
[n] Plumel, M.: Bull. Soc. chim. biol., 30:55, 1948; Fleury, P., Courtois, J., and Plumel, M.: Bull. Soc. chim. biol., 32:40, 1950.
[o] Lohmann, K.: Biochem. Ztschr., 203:172, 1928; Kurata, K.: J. Biochem. (Japan), 14:25, 1931; Ochiai, E.: Biochem. Ztschr., 253:185, 1932; Takahashi, H.: J. Biochem. (Japan), 16:447, 1932; Bamann, E., and Gall, H.: Biochem. Ztschr., 293:1, 1937; Da Cunha, D. P.: Compt. rend. Soc. de biol., 124:1023, 1937; Schäffner, A., and Krumey, F.: Ztschr. f. physiol. Chem., 255:145, 1938; Režek, A.: Chem. Abstr., 42:7808, 1948; Binkley, F., and Olson, C. K.: J. Biol. Chem., 186:725, 1950.
[p] Kitasato, T.: Biochem. Ztschr., 201:206, 1928.
[q] Bailey, K.: Biochem. J., 36:121, 1942; DuBois, K. P., and Potter, V. R.: J. Biol. Chem., 150:185 1943.
[r] Zeller, E. A.: Experientia, 4:194, 1948; Zeller, E. A.: Helvet. chim. acta, 33:281, 1950.
[s] Suzuki, U., Yoshimura, K., and Takaishi, M.: Bull. Coll. Agr. Tokyo Imp. Univ., 7:503, 1906–

In other cases their substrate and pH requirements are such that no suitable precipitation reactions have been developed for their demonstration. Enzymes with pH optima around the neutral point present especially great difficulties because, on the one hand, Ca salts cannot be used effectively below pH ± 8.5 and, on the other hand, the Pb salts of the substrates are not sufficiently soluble at a pH higher than ± 5.

Alkaline Phosphatases

Phosphatases with a pH optimum around 9 occur in most organs of almost all species examined. The largest group, that of the nonspecific enzyme(s), will hydrolyze any monoester of phosphoric acid and, in addition, nucleic acids. High polymer, native desoxyribose nucleic acid is not attacked[12–14] and must be depolymerized first if intended for use as a substrate. The rates of hydrolysis of various substrates vary over a wide range, and the optimal pH also depends on the nature of the substrate. As a rule, aromatic esters are hydrolyzed optimally at a higher pH (9.7–10) than aliphatic ones (8.1–9).[15] All enzymes of the group are activated by Mg. They are inhibited by cyanide (except hexosediphosphatase,[16] which is activated but cannot be demonstrated histochemically). Cysteine is also a strong inhibitor.[17]

Whether or not nonspecific alkaline phosphatase is a

12. Krugelis, E. J.: Biol. Bull., **90**:220, 1946.
13. Krugelis, E. J.: Genetics, **31**:221, 1946.
14. Ross, M. H., and Ely, J. O.: J. Cell. & Comp. Physiol., **34**:71, 1949.
15. King, E. J., and Delory, G. E.: Biochem. J., **33**:1185, 1939.
16. Gomori, G.: J. Biol. Chem., **184**:647, 1950.
17. Albers, H.: Ber. d. deutsch. chem. Gesellsch., **68**:1443, 1935; Hoffmann-Ostenhof, O., Moser, H., and Putz, E.: Experientia, **4**:352, 1948.

8; Plimmer, R. H. A.: Biochem. J., **7**:43, 1913; Rapoport, S., Leva, E., and Guest, G. M.: J. Biol. Chem., **139**:621, 1941; Courtois, J.: Bull. Soc. chim. biol., **30**:37, 1948.

[t] Zeller, E. A.: Enzymes as essential components of bacterial and animal toxins. In Sumner and Myrbäck's The enzymes I, **2**:986 (New York: Academic Press, 1950).

[u] Harris, D. L.: J. Biol. Chem., **165**:541, 1946; Feinstein, R. N., and Volk, M. E.: J. Biol. Chem., **177**:339, 1949.

[v] Neuberg, C., and Fischer, H. A.: Enzymologia, **2**:191, 241, 360, 1937–38; Frankenthal, L., Roberts, J. S., and Neuberg, C.: Exper. Med. & Surg., **1**:386, 1944.

[w] Ichihara, M.: J. Biochem. (Japan), **18**:87, 1933; Bredereck, H., and Geyer, E.: Ztschr. f. physiol. Chem., **254**:223, 1938; Lora Tamayo, M., and Martin Municio, A.: An. real Soc. españ. de fis. y quim., ser. B, **47**:149, 1951.

single enzyme or a group of enzymes with closely similar, although not identical, properties has been a matter of considerable argument. There is a large body of chemical data available in support of the theory of plurality.[7] Alkaline phosphatases of different sources may show marked differences in substrate preference and in their susceptibility to various activators and inhibitors. However, even if all the enzymes reported to exist do actually represent truly individual entities, the majority of them appear to be destroyed in the course of fixation and/or embedding. There are a considerable number of reports asserting that the localization of activity depends on the substrate used and that the differences observed are due to the presence of several enzymes;[12–14, 18] however, since the results have been obtained mostly under highly unfavorable conditions (prolonged incubation at low pH levels), the evidence cannot be accepted at face value. The slight variations due to different substrates may very well be considered the expression of differences in the rates of hydrolysis and in optimal pH values, the enzyme being identical in all cases (see p. 172). The writer found that under optimal or near-optimal conditions the pictures obtained with a large variety of substrates were indistinguishable from one another. Moog,[19] Ross and Ely,[14] Zorzoli and Stowell,[20] and others[21] have come to a similar conclusion. The only exception is 5-nucleotide: this substance is hydrolyzed by a specific enzyme. It should be emphasized specifically that

18. Deane, H. W., and Dempsey, E. W.: Anat. Rec., **94**:456, 1946; Dempsey, E. W., and Deane, H. W.: J. Cell. & Comp. Physiol., **27**:159, 1946; Dempsey, E. W., and Singer, M.: Endocrinology, **38**:270, 1946; Deane, H. W.: Am. J. Anat., **80**:321, 1947; Dempsey, E. W., and Wislocki, G. B.: Am. J. Anat., **80**:1, 1947; Süllmann, H.: Ztschr. f. Vitamin-, Hormon- u. Fermentforsch., **1**:374, 1947–48; Lagerstedt, S., and Stenram, U.: Acta anat., **10**:348, 1950; Newman, W., Feigin, I., Wolf, A., and Kabat, E. A.: Am. J. Path., **26**:257, 1950.

19. Moog, F.: Biol. Bull., **86**:51, 1944.

20. Zorzoli, A., and Stowell, R. E.: Anat. Rec., **97**:495, 1947.

21. Bevelander, G., and Johnson, P. L.: Anat. Rec., **104**:125, 1949; Hébert, S.: Arch. de biol., **61**:235, 1950.

results obtained with nucleic acids and with diesters of phosphoric and pyrophosphoric acid as substrates (in the case of the last two substrates, by the azo dye technique, p. 184) are indistinguishable from those seen with the use of glycerophosphate,[22] for example. There is no histochemical indication of the presence of diesterases or pyrophosphatases in paraffin-embedded animal tissues.

According to Emmel,[23] renal phosphatase and intestinal phosphatase in the mouse can be differentiated from each other by their markedly different sensitivities to cyanide and acid. His findings have been verified,[22] but, since they do not apply to species other than the mouse,[24] they must not be interpreted as an indication of a general difference between renal and intestinal phosphatase.

In view of the conflicting findings and opinions, the problem of the presence of several substrate-specific alkaline phosphatases in embedded tissues requires critical re-examination.

Friedenwald and Crowell[25] and Maengwyn-Davies and Friedenwald[26] found, by the use of various substrates and activators, that fresh unfixed tissues contain substrate-specific phosphatases which cannot be demonstrated in fixed tissues. This appears to be an important discovery which deserves thorough investigation.

Technical details.—

Freshness of the tissue is not a very important factor. Good results may be obtained with refrigerated tissue fixed as late

22. Gomori, G.: Unpublished.

23. Emmel, V. M.: Anat. Rec., **95**:159, 1946; Emmel, V. M.: *ibid.*, **96**:423, 1946; Emmel, V. M.: *ibid.*, **103**:445, 1949; Emmel, V. M.: J. Nat. Cancer Inst., **10**:1365, 1950; Emmel, V. M.: Proc. Soc. Exper. Biol. & Med., **75**:114, 1950; Emmel, V. M.: Anat. Rec., **106**:270, 1950.

24. Gomori, G.: Ann. New York Acad. Sc., **50**:968, 1950.

25. Friedenwald, J. S., and Crowell, J. E.: Bull. Johns Hopkins Hosp., **84**:658, 1949.

26. Maengwyn-Davies, G. D., and Friedenwald, J. S.: J. Nat. Cancer Inst., **10**:1379, 1950.

as 48 hours after removal, although there may be some blurring of the picture, owing to diffusion of the enzyme.

Freezing-drying appears to preserve the enzyme much better than chemical fixation and embedding; the difference is especially conspicuous in cases of low activity.

TABLE 3

Fixation	Percentage of Enzymatic Activity Preserved	
	Alkaline Phosphatase	Acid Phosphatase
Formalin	0–24[a] 40[b] 26[d]	±30[a] 90[b] 10[e] 55[d]
80 per cent alcohol	30[e] 65[f]	70[e] 11[e]
Absolute alcohol	75[f] 75[g]	15[f] 17[g]
Acetone	80[e] 75[f] 70[g] ±100[h]	±80[b] 25[f] 20[g] 22[e]

[a] Emmel, V. M.: Anat. Rec., **95**:159, 1946.

[b] Cappelin, M.: Bull. d'histol. appliq. à la physiol., **24**:155, 1947; Cappelin, M.: Monit. zool. ital., (suppl.) **56**:256, 1948.

[c] Rabinovitch, M., Junqueira, L. C., and Fajer, A.: Stain Technol., **24**:147, 1949.

[d] Seligman, A. M., Chauncey, H. H., and Nachlas, M. M.: Stain Technol., **26**:19, 1951.

[e] Cappelin, M.: Bull. d'histol. appliq. à la physiol., **24**:155, 1947.

[f] Doyle, W. L.: Proc. Soc. Exper. Biol. & Med., **69**:43, 1948.

[g] Stafford, R. O., and Atkinson, W. B.: Science, **107**:279, 1948.

[h] Cleland, K. W.: Proc. Linnean Soc. New South Wales, **75**:35, 1950.

All fixatives cause considerable inactivation of both alkaline and acid phosphatases. The effect of the procedures of fixation and embedding has been studied chemically by several workers, and the quantitative data are show in Table 3.

Cappelin[27] finds that fixation in chloroform (if chloroform can be called a fixative) preserves 98 per cent of the activity of both acid and alkaline phosphatases.

The discrepancy between the results of various workers is even more marked in reality than it would appear from

27. Cappelin, M.: Bull. d'histol. appliq. à la physiol., **24**:155, 1947; Cappelin, M.: Monit. zool. ital. (suppl.), **56**:256, 1948.

Table 3, since many of the figures quoted are actually averages, computed from widely divergent data. Some of the differences may be due to purely technical variations (temperature; length of exposure to the fixative; size of pieces used, and, consequently, rate of penetration by the fixative) and also to the different types of tissue used. The latter point is important, since the relative amounts of sensitive and resistant enzymes may vary considerably in different tissues. To mention only one example: while a large percentage of the alkaline phosphatase activity of liver tissue is due to unstable hexosediphosphatase, intestinal mucosa contains very little, if any, of this enzyme.

If the effect of various fixatives is tested in Coujard slides, purified intestinal phosphatase being used as the enzyme, alcohol and acetone are found to cause relatively little loss of activity (distinctly less than 50 per cent in 72 hours at 5° C.); neutralized formalin destroys over 75 per cent of the enzyme in less than 24 hours at room temperatue but has very little effect at icebox temperature.

Decalcification of tissues by the regular procedures will destroy all phosphatases. Lorch[28] and Greep, Fischer, and Morse[29] report that small pieces of bone can be decalcified, after alcohol fixation, in a citrate buffer of pH 4.4–5 (exposure, several days). After decalcification, the tissue is reactivated around pH 9 (barbital buffer), dehydrated, and embedded. To what extent the enzyme is preserved has not been determined, but enough activity remains to permit localization by the usual methods.

Frozen sections show a higher activity than embedded tissues (Danielli);[30] however, there is a danger of loss of enzyme by diffusion, since fixation in acetone or alcohol will not render the enzyme completely and irreversibly insoluble.

Embedding in paraffin causes a further loss in activity, esti-

28. Lorch, I. J.: Nature, **158**:269, 1946.

29. Greep, R. O., Fischer, C. J., and Morse, A.: Science, **105**:666, 1947; Greep, R. O., Fischer, C. J., and Morse, A.: J. Am. Dent. A., **36**:427, 1948.

30. Danielli, J. F.: J. Exper. Biol., **22**:110, 1946.

mated at 40–45,[31] 65,[32] and 75 per cent,[30] respectively. On the other hand, Cappelin[27] and Cleland[33] find that embedding causes very little inactivation. In Coujard experiments, embedding did not inactivate the slides noticeably except after formalin fixation (±50 per cent). No data are available on the effects of celloidin-embedding.

Paraffin blocks, in the writer's experience, retain their activity unchanged for over 10 years. Lison[34] finds that they may deteriorate in 2 months.

Cut ribbons can be kept in the icebox for many months; melted-on sections can be stored at room temperature for years without the slightest loss in activity. Danielli, Doyle,[31] and Lison, on the other hand, observed definite inactivation of the enzyme in stored paraffin sections.

1) *The calcium phosphate method.*—The first histochemical procedure was published by Takamatsu[35] in 1938, in a journal not readily accessible to Western readers. In 1939 the procedure was republished by Takamatsu[36] and simultaneously described independently by Gomori.[37] It was based on the principle that, if sections are incubated with glycerophosphate at an alkaline reaction in the presence of Ca ions, the phosphate ions liberated will be precipitated *in statu nascendi* (at the site of formation) as insoluble Ca phosphate. The latter is then transformed, in a second step, into metallic silver or black cobalt sulfide.

The composition of the incubating mixture may be varied within fairly wide limits without much difference in the final results. The pH of the solution should be between 9 and 9.8 (in the lower ranges in the case of aliphatic substrates, in the higher ranges in the case of aromatic ones). Below pH

31. Doyle, W. L.: Proc. Soc. Exper. Biol. & Med., **69**:43, 1948.
32. Stafford, R. O., and Atkinson, W. B.: Science, **107**:279, 1948.
33. Cleland, K. W.: Proc. Linnean Soc. New South Wales, **75**:35, 1950.
34. Lison, L.: Bull. d'histol. appliq. à la physiol., **25**:23, 1948.
35. Takamatsu, H.: Manshu Igaku Zasshi, **31**:34, 1938.
36. Takamatsu, H.: Tr. Soc. Path. Jap., **29**:492, 1939.
37. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **42**:23, 1939.

9 the intensity of the reaction rapidly declines; only sites of highest activities will be stained after short periods of incubation (up to 2 hours), and on greatly prolonged incubation diffusion artifacts may become very disturbing. Almost any buffer with a suitable pK can be used (NH_4Cl–NH_4OH; barbital; 2-amino-2-methyl-1,3-propanediol). Borax is better avoided because it inhibits the hydrolysis of glycerophosphate and of certain other substrates and because of its incompatibility with higher concentrations of Ca. The concentration of the buffer should be 0.05–0.1 M. The cheapest and easiest available substrate is glycerophosphate, any commercial brand of which can be used; the recommended concentration, 0.01–0.03 M. Instead of glycerophosphate, any phosphoric monoester, compatible with Ca at pH 9, can be used. In the case of unstable substrates, such as adenosinetriphosphate or acyl phosphates, supersaturation artifacts are unavoidable but can be recognized a such because they will be present even in inactivated sections.

The importance of the concentration of Ca ions has become appreciated only recently. It has been known for some time that nuclei will stain rather intensely around centers of high activity, and opinions have been expressed that this phenomenon is an artifact due to diffusion of Ca phosphate or of the enzyme itself (pp. 144–45). The experiments of Novikoff[38] and Gomori[39] have shown decisively that the alkaline phosphatase reaction of cell nuclei is due to the secondary adsorption of Ca phosphate only; the nuclei do not contain any enzyme either originally or by secondary adsorption (except possibly in cases of poor fixation). The latter point is also proved by the invariable lack of any nuclear reaction with the azo dye method (Lorch,[40] Novikoff, and Gomori). In the Ca-Co method, the staining of nuclei is due to the relatively low concentration of Ca ions in the incubating

38. Novikoff, A. B.: Science, **113**:320, 1951.
39. Gomori, G.: J. Lab. & Clin. Med., **37**:526, 1951.
40. Lorch, J.: Quart. J. Micr. Sc., **88**:159, 1947.

mixture (0.01–0.02 M, Gomori; 0.03 M, Takamatsu). If this concentration is increased to about 0.1 M, nuclear reaction and other diffusion artifacts are completely eliminated unless incubation is unduly prolonged. Higher concentrations of Ca inhibit phosphatase activity.

Ruyter[41] proposes the use of Mg in the presence of an ammonia buffer instead of Ca. The precipitate obtained will be magnesium ammonium phosphate. This method is not recommended, because the precipitate is rather coarsely crystalline and does not permit fine localization. However, the addition of a small amount of Mg (around 0.005 M) to the incubating medium is useful because of its activating effect.

Alkaline phosphatase is not too sensitive to minor variations in temperature; incubation at any temperature between 30° and 45° C. will do.

The length of incubation may be varied between wide limits, depending on the intensity of the reaction desired. However, it should be borne in mind that prolonged incubation, even under optimal conditions, favors diffusion artifacts (Danielli,[42] Gomori[39]). It would be difficult to establish a definite time limit beyond which it is not safe to go. In the writer's experience, after 4 or 5 hours false localizations become quite noticeable, and after 12–16 hours they may be widespread and intense. However, this complication need not arise except in special cases, since prolonging incubation beyond 2–3 hours is seldom, if ever, indicated.

Although the precipitate is clearly visible in polarized light,[43] much sharper pictures are obtained by any of the color reactions mentioned under "Calcium." The best pictures are obtained with the use of silver and cobalt salts. Dorfman and Epshtein[44] prefer the Turnbull blue method

41. Ruyter, J. H. C., and Neumann, H.: Biochim. et biophys. acta, **3**:125, 1949.

42. Danielli, J. F.: Nature, **165**:762, 1950.

43. Belanger, L. F.: Proc. Soc. Exper. Biol. & Med., **77**:266, 1951.

44. Dorfman, V. A., and Epshtein, S. M.: Doklady Akad. Nauk S.S.S.R., **72**:977, 1950.

(ferrous sulfate followed by ferricyanide). Bourne[45] suggests staining with alizarinesulfonate.

A very interesting method of visualization has been described by Dalgaard.[46] If glycerophosphate containing radioactive phosphorus is used, the precipitate can be demonstrated by its radioautogarph. The secondary introduction of radioactive lead into the precipitate is also possible.[47]

The sensitivity of the method, as determined by the Coujard method, is about 20–30 μM-units (0.5–1 Bodansky unit) per gram of active structure, the length of incubation being 1 hour. Proportionality holds for the period of between 2–3 minutes and 12 hours.

Sources of error.[48]—

a) False negative reactions (i.e., negative reactions in spite of the chemically verified presence of enzyme) will be obtained whenever the concentration of the enzyme is below the threshold of sensitivity of the method. Inactivation of the enzyme by technical error is an unlikely complication, provided that the method suggested is adhered to with reasonable accuracy. However, if for special reasons experimental conditions have to be changed considerably (use of low pH values, inhibitors, high ionic strength), negative results must be interpreted with caution.

b) False positive reactions were briefly mentioned on page 148. Pigments can be seen in unincubated sections; some of them can be identified by specific reactions (hemosiderin, ceroid, etc.). Preformed calcifications which will give reactions indistinguishable from true enzymatic ones can be removed before incubation by treating the sections for about 10 minutes with a citrate buffer of pH 4.5–5. The adsorption of cobalt salt on tissue protein is not likely to cause any trouble. Artifacts due to the spontaneous or bac-

45. Bourne, G.: Quart. J. Exper. Physiol., **32**:1, 1943.

46. Dalgaard, J. B.: Nature, **162**:811, 1948.

47. Barka, T., Szalay, S., Pósalaky, Z., and Kertész, L: Kisérletes orvostud., p. 1, 1951.

48. Gomori, G.: J. Lab. & Clin. Med., **35**:802, 1950.

terial decomposition of substrates were discussed earlier. Ruyter[41] finds that positive reactions may be obtained after prolonged incubation in alkaline solutions of Ca salts not containing any substrate. The writer was unable to duplicate this observation.

c) False localizations have been dealt with in detail (pp. 142 ff.).

Method recommended

Fixation and embedding (see pp. 138 ff.). It is advisable, although not strictly necessary, to protect slides with collodion.

Composition of the incubating mixture:

3 per cent (±0.1 M) solution of Na glycerophosphate	5–10 ml.
2 per cent (±0.2 M) solution of calcium chloride	20–25 ml.
10 per cent (±0.5 M) solution of magnesium chloride (or sulfate)	About 10 drops
Na barbital (powder)	1 knifepointful (0.5–1 g.)
Distilled water to make	50 ml.

Instead of barbital, other buffers (p. 221) may be used.

Should the mixture be turbid (presence of free phosphate in the substrate), it must be filtered before use.

Incubate sections around 37° C. for 1–4 hours.

Wash them under the tap for about 1 minute.

Immerse in a 1–2 per cent solution of any soluble cobalt salt (acetate, chloride, sulfate, nitrate) for 5 minutes.

Wash under the tap for 1–2 minutes.

Immerse in a dilute solution of colorless or light-yellow ammonium sulfide (a few drops to a Coplin jarful of distilled water) for 5 minutes.

Wash under the tap.

Counterstain as desired. Dehydrate and mount.

Sites of alkaline phosphatase activity appear in black.

2) *The azo dye method.*—The original form of this method was devised by Menten, Junge, and Green[49] in 1944; modi-

49. Menten, M. L., Junge, J., and Green, M. H.: J. Biol. Chem., **153**:471, 1944; Menten, M. L., Junge, J., and Green, M. H.: Proc. Soc. Exper. Biol. & Med., **57**:82, 1944.

fications were published by Manheimer and Seligman,[50] by Loveless and Danielli,[51] and by Gomori.[39] The first two methods use β-naphthyl phosphate as a substrate; Loveless and Danielli, a complicated azo dye phosphate; Gomori, α-naphthyl phosphate. The method of Gomori is the simplest; it employs only commerically available chemicals and does not require cooling by ice. The pictures obtained with it are remarkably sharp and much more detailed than those given by methods using β-naphthyl phosphate.

Method

Fixation and embedding as in the Ca-Co method.

Protection with collodion is better avoided because the membrane may be stained rather intensely by decomposition products.

Composition of the incubating mixture.—Dissolve about 10 mg. of Na α-naphthyl phosphate (available from Dajac Laboratories, 511 Lancaster Ave., Leominster, Mass.) in a few ml. of distilled water. Add a few ml. of a 4–5 per cent solution of borax ($Na_2B_4O_7 \cdot 10H_2O$), about 40 ml. of cool distilled water (not warmer than 20° C.), and a few drops of a 10 per cent solution of magensium chloride or sulfate. Stir into the mixture 20–50 mg. of any of the following diazonium salts: Blue B, Red RC, Bordeaux GP, Red G, or Fast Blue RR.

Incubate the slides for 10 to 30 minutes or until the desired intensity of staining is obtained. The slides may be removed from the incubating mixture and inspected under the microscope repeatedly. Mechanical stirring of the solution during incubation is highly advisable (p. 172).

Wash slides; counterstain with hematoxylin or with alum carmine; differentiate with acid alcohol (alcohol concentration, 70–80 per cent); wash again and either mount in glycerol-jelly or dehydrate and mount in balsam (p. 170). Sites

50. Manheimer, L. H., and Seligman, A. M.: J. Nat. Cancer Inst., **9**:181, 1948.

51. Loveless, A., and Danielli, J. F.: Quart. J. Micr. Sc., **90**:57, 1949.

of activity appear in shades listed in Table 1 (p. 171); background, yellowish.

The only possible source of error is the staining of enterochromaffin cells in a reddish shade, but only after formalin fixation.

The azo dye method (one or the red color variants), followed by Kóssa's technique for Ca, is very suitable for the simultaneous demonstration of preformed calcifications and of sites of phosphatase activity. It should supersede the older cobalt-sulfide–lead–acridine-red method.[52]

5-Nucleotidase

The existence of the enzyme 5-nucleotidase has been reported by Reis[53] and by Gulland and Jackson,[54] on the basis of chemical studies. The substrates of the enzyme are 5-nucleotides (muscle adenylic acid, inosinic acid, and, possibly, adenosinetriphosphoric acid). The pH optimum is around 7.8, but the enzyme is quite active even at pH 9.

The histochemical method[55] for 5-nucleotidase is very similar to the method for alkaline phosphatase; in fact, it may be identical with it except for the substrate. However, if sharp differentiation from alkaline phosphatase is desired, it is better to perform incubation at pH ± 8.3. At this pH, 5-nucleotidase is fully active, while the activity of alkaline phosphatase is only about one-third of the maximum. A slight disadvantage of this low pH is a tendency toward diffusion artifacts. It can be offset almost completely by a sufficiently high concentration of Ca ions.

The substrate used is muscle adenylic acid[56] (regular

52. Gomori, G.: Am. J. Path., **19**:197, 1943.

53. Reis, J.: Bull. Soc. chim. biol., **16**:385, 1934; Reis, J.: Enzymologia, **2**:110, 183, 1937–38; Reis, J.: *ibid.*, **5**:251, 1938–39; Reis, J.: *ibid.*, **2**:183, 1937–38; Reis, J.: Bull. Soc. chim. biol., **22**:30, 1940; Reis, J.: Biochem. J., **46**:xxi, 1950; Reis, J.: *ibid.*, **48**:548, 1951.

54. Gulland, J. M., and Jackson, E. M.: Biochem. J., **32**:597, 1938.

55. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **72**:449, 1949.

56. Available from the Schwarz Laboratories, 202 E. 44th St., New York 17; from Nutritional Biochemicals, Cleveland 28, and from the Sigma Chemical Co., 4648 Easton Ave., St. Louis 13.

adenylic acid is a 3-nucleotide). For histochemical purposes the cheaper grades appear to be just as good as the more expensive ones.

Method

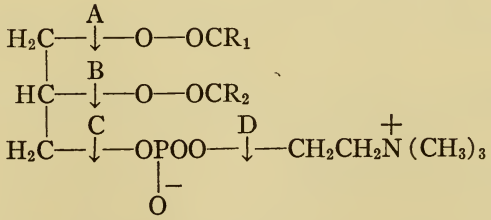
Dissolve about 20 mg. of muscle adenylic acid in 20 ml. of a 0.1–0.2 barbital or tris(hydroxymethyl)-aminomethane buffer of pH 8.3. Add 20 ml. of a 2 per cent $CaCl_2$ solution and a few drops of a 10 per cent $MgCl_2$ solution. Incubate sections for 2–5 hours at 37° C. Convert Ca phosphate precipitate into cobalt sulfide, as in the method for alkaline phosphatase.

The reaction obtained with 5-nucleotide is more widespread than that given by glycerophosphate or 3-nucleotides and includes, in addition to the sites of activity of nonspecific alkaline phosphatase, certain tracts in the central nervous system and the smooth muscle of blood vessels and of the urinary bladder.

The findings appear to be compatible with the assumption that, while nonspecific alkaline phosphatase will attack both glycerophosphate and the two nucleotides, 5-nucleotidase cannot hydrolyze substrates other than 5-nucleotide.

Lecithinase (Phospholipase)

Lecithin possesses 4 esteric linkages, and specific enzymes hydrolyzing each one of the linkages have been demonstrated.



The enzymes attacking bonds A and B belong among the lipases, while those attacking bonds C and D are phosphatases or, more exactly, phosphodiesterases. For detailed in-

formation and bibliography the reader is referred to the excellent review by Zeller.[57]

When paraffin sections of animal tissues are incubated with a "solution" of lecithin (or cephalin) in the presence of Ca ions around pH 7, no reaction whatsoever is observed, even on prolonged incubation (up to 12 hours).[58] At this pH, the Ca salts of fatty acids, if any were liberated, would precipitate promptly (see the Tween technique, p. 203), while Ca phosphate would not, unless the formation of phosphate ions were very rapid. At pH 9 a picture essentially identical with the pattern of distribution of alkaline phosphatase is obtained.[59] It should be mentioned here that Dempsey and Deane,[60] and Dempsey and Wislocki[61] did obtain positive reactions with lecithin around the neutral range but only after greatly prolonged incubation (24–72 hours). The artifacts produced under such conditions have been discussed (p. 176).

The findings seem to indicate that the enzymes responsible for the hydrolysis of bonds A and B cannot be demonstrated histochemically. This is surprising in view of the fact that such enzymes are known to occur in many animal tissues and that they are quite resistant to heat. The enzyme active at pH 9 and demonstrated histochemically acts either as lecithinase C or as lecithinase D. In the first case, the primary precipitate would be the Ca salt of phosphorylcholine; in the second case, Ca phosphatidate. Whether the primary precipitate is further hydrolyzed to Ca phosphate cannot be decided on the basis of data available.

The interesting point is that, just as in the case of the sim-

57. Zeller, E. A.: Enzymes as essential components of bacterial and animal toxins. In Sumner and Myrbäck's The enzymes (New York: Academic Press, 1951).

58. Gomori, G.: Unpublished.

59. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **70**:7, 1949.

60. Dempsey, E. W., and Deane, H. W.: J. Cell. & Comp. Physiol., **27**:159, 1946.

61. Dempsey, E. W., and Wislocki, G. B.: Am. J. Anat., **80**:1, 1947.

pler phosphodiesters (p. 177), the distribution of activity is identical with that of alkaline phosphatase. It appears very likely that the nonspecific enzyme is responsible for the histochemical reactions obtained with the use of phosphodiester substrates.

Acid Phosphatase

Phosphatases with very low pH optima have been described in fungi and in higher plants. A phosphatase with a pH optimum of ± 5 was found in the spleen and liver by Davies[62] and later in the prostate by Kutscher.[63] Acid phosphatases, as a rule, are not activated by Mg and almost invariably are greatly inhibited by fluoride. Enzymes of animal origin may show considerable differences in respect to their behavior toward inhibitors, as shown in Table 4.

TABLE 4

The Inhibition of Acid Phosphatases by Various Substances*

	Plasma	Red Cells	Adrenal	Bile	Prostate
Alcohol	3[a]	2[b]	ø[b]	4[b]	3[b, c]
Bile acid	ø[d]	3[d]	—	—	3[d]
Cu^{++}	—	4[e]	—	—	ø[e]
F^-	3–4[f, a]	ø–1[e, f, g, h]	—	—	4[e]
Formalin	—	4[b, i]	1[b]	2[b]	ø[b, i]
Tartrate	ø[d]	Only below pH 4.6; ø at pH 5[d, e]	—	—	4[d, e, j]

* ø indicates no inhibition; 4, maximum inhibition; 1–3, intermediate degrees; — indicates no data available.

[a] Gutman, E. B., and Gutman, A. B.: J. Clin. Investigation, 17:473, 1938.

[b] Abul-Fadl, M. A. M., and King, E. J.: J. Clin. Path., 1:80, 1948.

[c] Herbert, F. K.: Biochem. J., 38:23, 1944.

[d] Abul-Fadl, M. A. M., and King, E. J.: Biochem. J., 42:28, 1948.

[e] Abul-Fadl, M. A. M., and King, E. J.: Biochem. J., 45:51, 1949.

[f] Gutman, E. B., and Gutman, A. B.: Proc. Soc. Exper. Biol. & Med., 47:513, 1941.

[g] Behrendt, H.: Proc. Soc. Exper. Biol. & Med., 54:268, 1943.

[h] King, E. J., Wood, E. J., and Delory, G. E.: Biochem. J., 39:24, 1945.

[i] Abul-Fadl, M. A. M., and King, E. J.: J. Path. & Bact., 60:149, 1948.

[j] Seligman, A. M., and Manheimer, L. H.: J. Nat. Cancer Inst., 9:427, 1949; Seligman, A. M., Nachlas, M. M., Manheimer, L. H., Friedman, O. M., and Wolf, G.: Ann. Surg., 130:333, 1949.

62. Davies, D. R.: Biochem. J., **28**:529, 1934.

63. Kutscher, W., and Wolbergs, H.: Ztschr. f. physiol. Chem., **236**:237, 1935.

In addition to the differences tabulated, it appears that the ratio

$$\frac{\text{Hydrolysis of phenylphosphate}}{\text{Hydrolysis of glycerophosphate}}$$

is very much higher for the enzyme of red cells than for that of the plasma.[64]

The original histochemical method[65] for acid phosphatase utilizes the hydrolysis of glycerophosphate at pH 5 in the presence of Pb^{++} ions. Fixation in cold acetone and embedding in paraffin are recommended.

The results of this technique are not nearly so satisfactory or consistent as those of the method for alkaline phosphatase. A number of papers complaining of its capriciousness and unreliability have been published.[66–68]

When the causes of failures are analyzed, they are found to fall into six groups.

1. Inactivation of the enzyme in the course of fixation, embedding, storage, and incubation. There can be no doubt that only a relatively small fraction of the enzyme survives fixation and embedding. Inactivation may be fairly uniform or peculiarly patchy; the boundary between active and inactive areas is often quite sharp. Wolf, Kabat, and Newman[69] find that a layer at a certain distance from the surface of the block shows the best preservation of the enzyme. The writer is under the impression that embedding and not fixation (provided that cold acetone is used) is the main offender;

64. Behrendt, H.: Proc. Soc. Exper. Biol. & Med., **54**:268, 1943; Gutman, E. B., and Gutman, A. B.: Proc. Soc. Exper. Biol. & Med., **47**:513, 1941; Gutman, E. B., and Gutman, A. B.: J. Clin. Investigation, **17**:473, 1938; Herbert, F. K.: Biochem. J., **38**:23, 1944; King, E. J., Wood, E. J., and Delory, G. E.: Biochem. J., **39**:24, 1945.

65. Gomori, G.: Arch. Path., **32**:189, 1941.

66. Moog, F.: Proc. Nat. Acad. Sc., **29**:176, 1943.

67. Hard, W. L., and Lassek, A. M.: J. Neurophysiol., **9**:121, 1946.

68. Montagna, W., Noback, C. R., and Zak, F. G.: Am. J. Anat., **83**:409, 1948; Tissières, A.: Acta anat., **5**:224, 1948.

69. Wolf, A., Kabat, E. A., and Newman, W.: Am. J. Path., **19**:423, 1943.

especially a high temperature of the paraffin oven and long exposure of the tissue to hot paraffin seem to be harmful.

According to several authors (Hard and Lassek,[67] Doyle,[70] Goetsch and Reynolds[71]), the enzyme is gradually inactivated on storage of paraffin blocks, ribbons, or mounted sections.

Doyle asserts that lead ions inhibit the enzyme very markedly (about 85 per cent). Using the regular histochemical substrate mixture, the writer found about 35 per cent inhibition by lead in test-tube experiments.

2. The use of unfixed or partially fixed, unembedded tissue. Frozen sections may show a relatively high activity, but the affinity of undenatured proteins to lead salts is likely to produce false positive reactions. Lead adsorbed by paraffin-embedded tissues can be washed out readily by dilute acetic acid (exception under 4); however, frozen sections of unfixed or acetone-fixed tissues will hold lead so stubbornly that even prolonged washing in strong acetic acid cannot remove it completely. Formalin-fixed protein has much less affinity to lead.

3. Failure to use an acid rinse after incubation. Even denatured proteins may adsorb some lead from the substrate mixture but will release it easily when rinsed in dilute acetic acid. The acid rinse is an important step; it removes protein-bound lead but leaves enzymatically produced lead phosphate untouched. If it is omitted (as it apparently was by Newman, Kabat, and Wolf[72] and by Takeuchi and Tanoue[73]), the result will be a nonenzymatic staining of the background, mainly of nuclei.

4. Certain structures such as axons retain a high affinity to lead even after paraffin-embedding; in fact, axons can be

70. Doyle, W. L.: Proc. Soc. Exper. Biol. & Med., **69**:43, 1948.
71. Goetsch, J. B., and Reynolds, P. M.: Stain Technol., **26**:145, 1951.
72. Newman, W., Kabat, E. A., and Wolf, A.: Am. J. Path., **26**:489, 1950.
73. Takeuchi, T., and Tanoue, M.: Kumamoto M. J., **4**:41, 1951.

stained by lead impregnation (Mallory[74]). Nonenzymatic staining of axons by lead can easily be mistaken for an enzymatic reaction.[75] Control sections with either inactivated (by Lugol's solution or by boiling water) tissue or with active tissue incubated in the presence of 0.005 M fluoride must be used to detect nonenzymatic impregnation; it may be extremely difficult or impossible to determine whether the picture obtained is not a composite of both enzymatic and nonenzymatic staining.

5. Nonoptimal composition of the incubating mixture. The effect of the composition of the substrate mixture on the constancy of the results has been studied by Gomori, who found that the ratio between the concentrations of buffer and substrate is an important factor. An unduly high concentration of buffer will greatly reduce the sensitivity of the method, thereby giving rise to "all or none" phenomena. The original formula is definitely nonoptimal, and there can be no doubt that some of the failures obtained with it are due to this fact.

6. Undertermined factors. This group includes the queerest types of failure, unexplainable by any of the causes enumerated. To mention only a few examples: sections cut from a single block stain uniformly one day but utterly refuse to stain the next day, although the incubating mixture is prepared in exactly the same way both times; out of a ribbon of several serial sections, mounted on one slide, one or two stain exceedingly poorly while the majority show an excellent reaction.

Fixation of thin blocks in cold acetone, rapid embedding at a temperature not exceeding 56° C., and the use of recently cut sections and of the correct substrate mixture will produce good results in a vast majority of instances; however, occasional unexplainable failures cannot be eliminated completely.

74. Mallory, F. B.: Am. J. Path., **12**:569, 1936.

75. Bartelmez, G. W., and Bensley, S. H.: Science, **106**:639, 1947; Heinzen, B.: Anat. Rec., **98**:193, 1947; Lassek, A. M.: Stain Technol., **22**:133, 1947.

1) *The lead method.*[76]—Composition of the substrate mixture: In 500 ml. of a 0.05 M acetate buffer of pH 5 dissolve 0.6 g. of lead nitrate (about 0.003 M) and add 50 ml. of a 3 per cent (about 0.1 M) solution of Na glycerophosphate. The mixture will become turbid; the degree of turbidity depends on the percentage of β-isomer contained in the brand of glycerophosphate used. A mixture of about equal parts of the two isomers will cause much less turbidity than the more commonly sold mixtures containing around 75 per cent of β-salt. Keep the solution in the incubator at 37° C. for 24 hours, filter it. Add a small amount (about 5 per cent) of distilled water to the filtrate to prevent precipitation on evaporation. The mixture is ready for use and will keep in the icebox for months. If it becomes turbid, it should be discarded.

Carry sections through xylene and alcohols to water. Collodion coating of the sections is advisable; it appears to prevent loss of enzyme by diffusion (Doyle[70]), especially at sites of low activity, although it may cause some patchiness of the reaction (Goetsch and Reynolds[71]). Rinse slides thoroughly in distilled water.

Incubate in the substrate mixture around 37° C. for 1–24 hours. Human prostate usually requires 1–2 hours of incubation, other tissues 6–8 hours or more. Sites of activity will become a chalky white from the deposition of lead phosphate. Rinse slide first in distilled water, then for a minute or so in 1–2 per cent acetic acid and once more in distilled water. Immerse slide in a dilute solution of ammonium sulfide (a few drops to a Coplin jarful of distilled water) for about 2 minutes. Wash under the tap. Counterstain as desired. Dehydrate in alcohols; clear in gasoline or tetrachloroethylene, and mount in clarite or some similar resin dissolved in the same solvents. Toluene or xylene should not be used; they will cause some fading of the stain. Sites of activity are indicated by the dark brown-black precipitate of PbS.

76. Gomori, G.: Stain Technol., **25**:81, 1950; Wang, K. J.: Chinese J. Physiol., **17**:317, 1950.

For special purposes the method can be modified by changing the pH of the solution or by using other substrates. Above pH 6 the activity of alkaline phosphatase rapidly increases, and one may obtain combination pictures of the distributions of acid and alkaline phosphatase. The choice of substrates is rather limited because the lead salts of most phosphoric esters are very insoluble at pH 5 or higher. Resorcinol phosphate and adenosinetriphosphate are suitable substrates; they give pictures which differ more or less markedly from those obtained with glycerophosphate.[76] This may be an indication of the existence of more than one acid phosphatase in the tissues, but the point will require further investigation. Abolinš[77] finds that the pattern of distribution of activity in the anterior pituitary varies with the pH of the substrate solution, and he attributes the differences to the presence of several enzymes.

Sources of error are the same as in the method for alkaline phosphatase, with the addition of impregnation artifacts of nerve tissue.

The sensitivity of the method (Coujard's method) is about the same as that of the technique for alkaline phosphatase.

2) *The azo dye method.*—Seligman and Manheimer[78] recommend α-naphthyl phosphate as a substrate, in the presence of diazotized aminoanthraquinone. Sites of activity are shown in a reddish-brown shade. This method is not recommended because of unavoidable gross diffusion artifacts.

Phosphamidase

An enzyme hydrolyzing phosphamides has been described in animal and plant tissues.[79] In animals its natural substrates are probably phosphocreatine, phosphoarginine, and possibly some other less-well-known compounds possessing an N-P bond.

77. Abolinš, L.: Nature, **164**:455, 1949.

78. Seligman, A. M., and Manheimer, L. H.: J. Nat. Cancer Inst., **9**:427, 1949.

79. Waldschmidt-Leitz, E., and Köhler, F.: Biochem. Ztschr., **258**:360, 1933; Ichihara, M.: J. Biochem. (Japan), **18**:87, 1933; Bredereck, H., and Geyer, E.: Ztschr. f. physiol. Chem., **254**:223, 1938.

For the histochemical demonstration of phosphamidase the substrate used is phophoric acid *p*-chloroanilide (*p*-chloroanilidophosphonic acid). This compound is relatively stable and easier to prepare than the other phosphamides. There is some doubt concerning its correct formula; Rorig[80] believes that the compound synthesized by the method of Otto[81] is actually a derivative of diamidophosphoric acid. It appears that different batches prepared by the same method are not necessarily identical in composition.

The data on the optimal conditions of activity (pH, activators, etc.) of this enzyme are rather vague. The histochemical method to be described has been worked out by the method of trial and error only. Typical pictures are obtained only in the pH range between 5 and 6; in the alkaline range, chloroanilidophosphonic acid as a substrate gives distribution patterns indistinguishable from the regular alkaline phosphatase reaction. Takamatsu and Sho[82] have used phosphocreatine as a substrate at an alkaline pH; the description of the results obtained is not clear enough to permit the drawing of conclusions.

Method[83]

Fixation in acetone or alcohol. Sections should not be coated with collodion.

Dissolve 1 g. *p*-chloroanilidophosphonic acid[84] in an ice-cold mixture of 1 ml. of concentrated ammonia and 40 ml. of distilled water with vigorous shaking. The substance usually does not dissolve completely. Filter. Add 2 drops of a 0.5 per cent phenolphthalein solution. Titrate back with M acetic acid to a barely perceptible pink shade (about 3 ml. of acid will be required). Fill up to 50 ml. with distilled water. Keep in the icebox. For use, add 0.8–1 ml. of a 5 per

80. Rorig, K.: J. Am. Chem. Soc., **71**:3561, 1949.
81. Otto, P.: Ber. d. deutsch. chem. Gesellsch., **28**:616, 1895.
82. Takamatsu, H., and Sho, E.: Tr. Soc. Path. Jap., **32**:90, 1942.
83. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **69**:407, 1948.
84. Obtainable from Dajac Laboratories, 511 Lancaster Ave., Leominster, Mass.

cent solution of lead nitrate to 50 ml. of a 0.05 M maleate buffer of pH 5.5–5.6; shake until the initial white precipitate dissolves. Add a few drops of a 0.2 M solution of $MnCl_2$ and 1.5–2 ml. of the phosphonate stock solution. If the mixture becomes turbid, place it in the paraffin oven and keep it there for 10–30 minutes or until the turbidity settles. Filter it into a Coplin jar. Incubate sections for 12–24 hours at 37° C. Since the substrate is not entirely stable at pH 5.5 but decomposes slowly into free phosphate and *p*-chloroaniline, the Coplin jar must be supported in an inclined position, with the tissues facing downward, to avoid, as far as possible, the indiscriminate precipitation of lead phosphate all over the tissues. This maneuver will cause the heavy precipitate to settle on the back surface of the slides.

Remove slides from the incubating mixture, wipe precipitate from back surface. Rinse them thoroughly in distilled water. Remove superficial precipitate by moving the slides around in a 0.1 M citrate buffer of pH 4.5–5. As soon as the glass appears completely clear around the tissue, rinse slides under the tap. This is the most delicate step in the entire procedure. Insufficient differentiation will leave a coarse black precipitate in the finished section, while overdifferentiation may remove part or all of the lead phosphate deposited by enzymatic action.

Treat slides with ammonium sulfide, etc., as in the method for acid phosphatase.

In its present form the method is not entirely dependable. The difficulties are essentially the same as with the acid phosphatase technique (lack of uniformity in staining, sometimes even in the case of consecutive serial sections; unexplained failures to get a positive reaction).

Moderate amounts of the enzyme are found in practically all animal tissues; very high concentrations can be demonstrated in the vast majority of malignant epithelial neoplasms. As a rule, the intensity of the reaction roughly parallels the degree of histological malignancy. Polyps of the

colon form an interesting exception; they all react intensely, even though they may be completely benign histologically.

Phosphorylase

Enzymes catalyzing the reaction

Polysaccharide + inorganic phosphate $\rightleftarrows$ glucose-1-phosphate

(where the polysaccharide may be either starch of glycogen) have been described by Cori and Cori,[85] Kiessling,[86] and Hanes.[87] If the reaction is started from the right side, a trace of polysaccharide is needed as a primer; the equilibrium of the reaction depends mainly on the pH. At pH $\pm$ 5.7, the equilibrium is shifted to the left; at pH 7–7.6, to the right.

Successful attempts at the demonstration of phosphorylase have been made by Yin and Sun[88] and by Cobb.[89] Yin and Sun used sections of starch-free, water-soaked sections of soybean; Cobb used paraffin sections of frozen-dried cartilage from which glycogen had been removed by saliva. In both cases the tissues were incubated with glucose-1-phosphate at pH $\pm$6 for 30 minutes to 6 hours; starch and glycogen, respectively, were demonstrated by specific stains. This method deserves further investigation, especially in respect to the optimal conditions of the reaction.

Zymohexase (Aldolase)

Allen and Bourne[90] have published a method for the demonstration of zymohexase. It is based on the fact that the

85. Cori, C. F., and Cori, G. T.: Proc. Soc. Exper. Biol. & Med., **34**:702, 1936; Cori, C. F. and Cori, G. T.: *ibid.*, **36**:119, 1937; Cori, C. F.: Endocrinology, **26**:285, 1940; Cori, G. T., and Cori, C. F.: J. Biol. Chem., **135**:733, 1940.

86. Kiessling, W.: Biochem. Ztschr., **298**:421, 1938.

87. Hanes, C. S.: Proc. Roy. Soc. London, B, **128**:421, 1940; Hanes, C. S.: *ibid.*, **129**:174, 1940.

88. Yin, H. C., and Sun, C. N.: Science, **105**:650, 1947.

89. Cobb, J. D.: The morphological distribution of glycogen and glycoproteins in the cells and extracellular materials of growing bones (thesis, University of Illinois, 1949).

90. Allen, R. J. L., and Bourne, G. H.: J. Exper. Biol., **20**:61, 1943.

enzyme splits hexosediphosphate into two molecules of triose phosphate. The latter hydrolyzes spontaneously at an alkaline reaction, and the phosphate liberated is visualized much as in the regular method for alkaline phosphatase. Iodoacetate and fluoride are added to the incubating mixture to prevent the dismutation of triose phosphate and the hydrolysis of hexosediphosphate by alkaline phosphatase, respectively. Frozen sections (whether fixed or unfixed, not clear from the text) must be used, because paraffin sections give no reaction.

This method is open to criticism on several counts. Zymohexase itself is quite soluble and cannot be expected to remain *in situ* under the conditions of the method. But the main objection is this: even if it were granted that hexosediphosphate will be attacked by only zymohexase under the conditions specified (and this is certainly not true, since regular alkaline phosphatase hydrolyzes hexosediphosphate quite readily and is not inhibited by fluoride), the second step, namely, that of the spontaneous dephosphorylation of triose phosphate, is far too slow to permit localization, since triose phosphate is highly diffusible. There can be no doubt that its decomposition will take place almost quantitatively in the ambient fluid and not in the tissue.

The illustrations given in the original paper do not correspond to the distribution of regular alkaline phosphatase. However, it is impossible to tell just what is demonstrated by the method. It is quite likely that some undetermined unstable phosphatase, intolerant to embedding, is responsible for the reaction.

Sulfatase

Extracts of plant and animal tissues hydrolyze a variety of sulfuric esters, such as chondroitinsulfuric acid,[91] sulfates of

91. Neuberg, C., and Hoffmann, E.: Biochem. Ztschr., **234**:345, 1931.

indoxyl,[92] phenols,[93] estrogens,[94] sugars,[95] and thioglucosides.[96] There seem to be four different enzymes involved, with rather sharp substrate specificities. For further details the reader is referred to Fromageot's excellent review article.[97]

Sulfatases hydolyzing sulfates of phenols[97] and of thioglucosides[96] have been demonstrated in the tissues of higher animal species. The enzymes are quite resistant to dehydration by acetone.[98] The concentration of enzyme is very low (maximum, 50–60 μM of substrate hydrolyzed per gram of tissue and per hour;[99] in most cases activity is very much lower, usually around 1 per cent of the phosphatase activity).[100]

Theoretically, either the sulfate or the phenolic component could be demonstrated by histochemical methods. The sulfate method was tried with nitrophenyl sulfate as a substrate,[101] without any success, although this compound is hydrolyzed at a much higher rate than other esters. Ohara and Kurata[102] have reported positive results by the lead sulfate technique, using phenyl sulfate or 8-hydroxyquinoline sulfate as substrates. Another method by the same authors demonstrates the sulfate ion by precipitating it with benzidine; the latter is then demonstrated with β-naphthoquinone sulfonate. These methods seem to work with acetone-fixed,

92. Derrien, M.: Bull. Soc. chim. France, ser. IV, **9**:110, 1911.
93. Neuberg, C., and Kurono, K.: Biochem. Ztschr., **140**:295, 1923.
94. Cohen, H., and Bates, R. W.: Endocrinology, **44**:317, 1949.
95. Soda, T., and Hattori, C.: Proc. Imp. Acad. Tokyo, **7**:269, 1931.
96. Neuberg, C., and Wagner, J.: Ztschr. f. d. ges. exper. Med., **56**:334, 1927.
97. Fromageot, C.: Ergebn. d. Enzymforsch., **7**:50, 1938.
98. Neuberg, C., and Simon, E.: Biochem. Ztschr., **156**:365, 1925.
99. Huggins, C., and Smith, D. R.: J. Biol. Chem., **170**:391, 1947.
100. Rosenfeld, L.: Biochem. Ztschr., **157**:434, 1925; Hommerberg, C.: Ztschr. f. biol. Chem., **200**:69, 1931.
101. Gomori, G.: Unpublished.
102. Ohara, M., and Kurata, Y.: Igaku to Seibutsugaku, **16**:213, 1950.

unembedded tissue only. Seligman and co-workers[103] have used the sulfate of 6-bromo- and 6-benzoyl-β-naphthol on frozen sections of tissues fixed briefly in formalin. A long incubation (24 hours) is required. Human tissues do not attack the benzoyl compound. No detailed description of the method was given.

Esterases

The term "esterase" will be used in the sense of enzymes hydrolyzing esters of carboxylic acids.

Esterases obtained from various sources and by various procedures may exhibit markedly different enzymatic properties, especially with respect to substrate specificity and to sensitivity to activators and inhibitors. Although the classification of esterases is still a partly controversial issue, it is generally agreed that they can be divided into two large groups: namely, aliesterases,[104] hydrolyzing esters of N-free alcohols, and cholinesterases, hydrolyzing esters of choline. The aliesterases are subidivided into lipases, which preferentially split fats and oils, and esterases, the substrates of which are simpler esters of monohydric alcohols. This classification is not complete; it includes only the more widespread and thoroughly investigated enzymes which are also of histochemical interest. Table 5 shows the most important biochemical differences between esterases.

It should be made clear that all the differences mentioned are relative rather than absolute and that there are many examples of overlapping between enzyme types. While some lipases possess all the features enumerated under "Lipase" and some esterases all the features of "Esterase," some enzymes occupy an intermediate position, in that in some respects they behave like a lipase and in others like an esterase.

103. Seligman, A. M., Nachlas, M. M., Manheimer, L. H., Friedman, O. M., and Wolf, G.: Ann. Surg., **130**:333, 1949; Seligman, A. M., and Nachlas, M. M.: Cancer Research, **10**:240, 1950.

104. Richter, D., and Croft, P. G.: Biochem. J., **36**:746, 1942.

TABLE 5

Biochemical Differences between Esterases

I. Aliesterases (substrates: esters of n-free alcohols)

	A. Lipase	B. Esterase
1. Substrate preferences:		
a) Chain length of fatty acid[a, b]	Long (>12)	Short (<12)
b) Branching of chain of fatty acid[a]	Straight chain	Iso chain
c) Aliphatic or aromatic nature of fatty acid[a]	Aliphatic	Aromatic
d) Nature of alcohol moiety	Glycerol	Monohydric alcohols
e) Rates of hydrolysis of nitrophenol esters of C_2–C_5 fatty acids[c]	2<3<4<5	2<3>4>5
f) Optical isomers[d, e]	The two types of enzymes often favor opposite optical isomers in an unpredictable way.	
2. Activators and inhibitors:		
a) Quinine[f, g, h, i]	Inhibition	No effect
b) Arsanilic acid[f, g, i]	No effect	Inhibition
c) Fluoride[j, k, l]	Slight inhibition	Marked inhibition
d) Bile acids[d, k, l]	Activation	Inhibition

II. Cholinesterases (substrates: esters of choline)

	A. So-called True or Specific Cholinesterase	B. So-called Pseudo- or Nonspecific Cholinesterase[m]
1. Substrate preferences[n]	Acetylcholine, mecholyl	Other choline esters
2. Optimal substrate concentration[o]	Low ($\pm 10^{-3.5}$ M)	High ($\pm 10^{-2}$ M)
3. Selective inhibitors[p]	Nitrogen mustard	Diisopropylfluorophosphate, percaine[q]

[a] Terroine, E. F.: Ann. sci. nat., zool., X[e] sér., 4:1, 1942.

[b] Schønheyder, F., and Volqvartz, K.: Enzymologia, 11:178, 1944.

[c] Huggins, C., and Moulton, S. H.: J. Exper. Med., 88:169, 1948.

[d] Willstätter, R., and Memmen, F.: Ztschr. f. physiol. Chem., 138:216, 1924.

[e] Ammon, R.: Fermentforsch., 11:459, 1929–30; Rona, P., and Ammon, R.: Ergebn. d. Enzymforsch., 2:50, 1933.

[f] Rona, P., and Pavlović, R.: Biochem. Ztschr., 130:225, 1922.

[g] Rona, P., and Pavlović, R.: Biochem. Ztschr., 134:108, 1923.

[h] Rona, P., and Takata, M.: Biochem. Ztschr., 134:118, 1923.

[i] Rona, P., and Haas, H. E.: Biochem. Ztschr., 141:222, 1923.

[j] Kastle, J. H., and Loevenhart, A. S.: Am. Chem. J., 24:491, 1900; Loevenhart, A. S., and Peirce, G.: J. Biol. Chem., 2:397, 1906–7.

[k] Nachlas, M. M., and Seligman, A. M.: J. Biol. Chem., 181:343, 1949.

[l] Seligman, A. M., Nachlas, M. M., and Mollomo, M. C.: Am. J. Physiol., 159:337, 1949.

[m] Glick, D.: Science, 102:100, 1945.

[n] Nachmansohn, D., and Rothenberg, M. A.: Science, 100:454, 1944.

[o] Mendel, B., Mundell, D. B., and Rudney, H.: Biochem. J., 37:473, 1943; Augustinsson, K. B.: Nature, 162:194, 1948.

[p] Mendel, B., and Hawkins, R. D.: Biochem. J., 41:22, 1947; Adams, D. H., and Thompson, H. S.: Biochem. J., 42:170, 1948; Adams, D. H.: Biochim. et biophys. acta, 3:1, 1949.

[q] Zeller, E. A., and Bissegger, A.: Helvet. chim. acta, 26:1619, 1943.

The substrate specificity of even the two large groups (aliesterases and cholinesterases) is not an absolute one; actually, both types will attack choline and noncholine esters.[105–7] The sharpest difference between any two groups of the system is in the sensitivity of esterases toward eserine and similar alkaloids. Cholinesterases are inhibited by concentrations of 10^{-6} M or less,[104, 106] while aliesterases are insensitive to as much as 10^{-3} M.[108] It is impossible to go into the details of the highly complex problem of the specificity of esterases; it is suggested that the interested reader study the original articles referred to.

I. ALIESTERASES

A suitable histochemical substrate for esterases must satisfy the following three conditions: (1) It must be sufficiently soluble in water, because esterases do not work in nonaqueous media. Kurata and Hoso[109] did get positive results with a suspension of olive oil; however, the method is very insensitive, showing only sites of extremely high activity. (2) It must by hydrolyzable by enzymes. (3) One of the split products must be precipitable *in statu nascendi.* Up to 1945 it appeared as if the three conditions were mutually exclusive. The first substrates to be used histochemically[110] were the Tweens, which are long-chained (C_{12}–C_{18}) fatty acid esters of sorbitan or mannitan, the remaining hydroxyl groups of which are etherified with ethylene oxide chains of varying lengths. These substances are water-soluble, readily attacked by esterases, and the fatty acid liberated can be trapped in the form of insoluble Ca soaps. Esters of poly-

105. Stedman, E., Stedman, E., and White, A. C.: Biochem. J., **27**:1055, 1933.

106. Easson, L. H., and Stedman, E.: Biochem. J., **31**:1723, 1937.

107. Mendel, B., and Rudney, H.: Biochem. J., **37**:59, 1943; Holton, P.: Biochem. J., **43**:13, 1948; Zeller, E. A.: Helvet. physiol. et pharmacol. acta, **6**:C36, 1948; Whittaker, V. P.: Biochem. J., **44**:46, 1949; McNaughton, R. A., and Zeller, E. A.: Proc. Soc. Exper. Biol. & Med., **70**:165, 1949.

108. Huggins, C., and Moulton, S. H.: J. Exper. Med., **88**:169, 1948.

109. Kurata, Y., and Hoso, M.: Igaku to Seibutzugaku, **18**:103, 1951.

110. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **58**:362, 1945.

ethylene glycols, marketed under various trade-names, have essentially similar properties.

The other type of substrate are esters of naphthols[111] (acetates or benzoates). The naphthol liberated is demonstrated by azo-coupling.

The best fixation for esterases and lipases is acetone, but alcohol is almost equally good. Relatively short (12–24 hours) fixation in 10 per cent formalin (preferably neutralized and at icebox temperature) is permissible, although, compared with acetone-fixed tissues, 50–75 per cent of the enzyme will be destroyed by it.

A. *The Tween technique.*—

Most of the lauric, palmitic, stearic, oleic, ricinoleic, or mixed esters available on the market are usable, provided that they are soluble in water. The rate of hydrolysis drops with increasing chain length, laurates being the fastest and stearates the slowest substrates. However, the crystal size of the precipitate also decreases with increasing chain length, and localization is far more accurate with the slow stearates than with the faster laurates, which produce rather coarse crystals.[112] The following substrates are recommended for use:

Saturated subtrates:

1. Stearates
   Tween 60 (Atlas Powder Co., Wilmington, Del.)
   G-9096-CJ (Atlas)
   G-2151 (Atlas)
   Product No. 81 (Onyx Oil and Chemical Co., Jersey City, N.J.)
2. Palmitate
   Tween 40 (Atlas)
3. Laurate
   Tween 20 (Atlas)

111. Nachlas, M. M., and Seligman, A. M.: J. Nat. Cancer Inst., **9**:415, 1949; Nachlas, M. M., and Seligman, A. M.: Anat. Rec., **105**:677, 1949.

112. Gomori, G.: Methods of study for tissue lipase. In Menstruation and its disorders (Springfield, Ill.: C. C. Thomas, 1950).

Unsaturated substrates:

1. Oleates
   Tween 80 (Atlas)
   G-2144 (Atlas)
   G-9446-N (Atlas)
   G-7627-DJ (Atlas)
   Neutronyx R (Onyx)
2. Ricinoleates (these are especially slow substrates)
   G-6486 (Atlas)
   Antarox B 290 (General Aniline and Film Corp.)
3. Mixed linseed oil esters
   G-9926-T (Atlas)

The saturated compounds are general substrates for lipase-esterase; they are hydrolyzed by the enzymes of many organs. The unsaturated substrates are attacked only by the enzymes of the pancreas and of the chief cells of the mouse stomach[113] ("true lipase") and are left practically untouched by those of other tissues. Whether the faint reactions obtained with these substrates in the intestine, liver, kidney, etc., are due to a slight activity of the enzyme toward the unsaturated ester or to the invariable presence of saturated esters (as impurities) in the commercial unsaturated compounds cannot be decided, because purified Tweens containing exclusively unsaturated fatty acids are not available.

The quality of the Tweens and similar substances varies considerably from batch to batch. Some batches are pure enough to be used directly, others will develop turbidity in the presence of Ca salts, thus indicating the presence of free fatty acid. It is advisable to purify them for histochemical use. The following routine is recommended:

Dissolve about 5 per cent of Tween in a 0.1 M tris (hydroxymethyl)-aminomethane or tris-maleate buffer of pH 7–7.4, add about 0.5 per cent of $CaCl_2$ and a few crystals of camphor (*important:* Tweens are excellent nutrient media for many microörganisms!). Incubate at 37° C. for 24–48 hours; filter through a fritted glass plate or a Seitz asbestos filter.

113. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **72**:697, 1949.

The filtered stock solution will keep in the refrigerator for many months.

Method

Collodion protection is useful but is permissible only in the case of saturated substrates; the unsaturated ones penetrate the membrane very poorly.

To about 45 ml. of 0.05 M tris(hydroxymethyl)-aminomethane or tris-maleate or barbital buffer of pH 7–7.4 add 3 ml. of a 2 per cent solution of $CaCl_2$, 1 ml. of 5 per cent Tween stock solution, and a few crystals of camphor. Incubate slides at 37° C. for 8–24 hours. Rinse slides in distilled water and immerse them in a 1–2 per cent solution of lead nitrate for 10 minutes at the temperature of the paraffin oven, to transform the Ca soaps into Pb soaps. Rinse slides in repeated changes of distilled water for 5–10 minutes and immerse them for about 5 minutes in a dilute solution of light-yellow ammonium sulfide. Wash under the tap, counterstain as desired, dehydrate in alcohols. Clear in gasoline or tetrachloroethylene; mount in synthetic balsam dissolved in one of the clearing agents mentioned. Xylene will cause rapid fading of the stain. Sites of lipase-esterase activity in a golden-brown shade.

Two minor drawbacks of the method should be mentioned. First, in some cases there is considerable cytolysis in the tissue, especially around areas of high activity, making counterstaining unsatisfactory. Treatment of the slide with 10 per cent formalin for 10–30 minutes before incubation will help to minimize this complication, without causing any appreciable loss in enzymatic activity. Secondly, a certain amount of diffusion of the enzyme (especially in the pancreas) appears to be unavoidable; specks of loose brown precipitate will almost always be found scattered around sites of high activity. The formalin treatment just mentioned will greatly reduce its amount but will rarely succeed in eliminating it completely. Most of the precipitate is formed

within the collodion membrane itself and can be washed off together with the latter by acohol-ether before clearing the section.

The hydrolysis of Tweens is susceptible to the effects of activators and inhibitors of esterase-lipase, such as bile salts, quinine, arsanilate, etc.[114]

The sensitivity of the Tween method (determined by Coujard's technique) is ±7 μM of acid liberated in 24 hours and per gram of active structure. Stearates are hydrolyzed at about one-third of the rate of laurates.

B. *The azo dye technique.*—

The first azo dye method for esterase was published by Nachlas and Seligman in 1949;[111] it was based on the enzymatic hydrolysis of β-naphthyl acetate in the presence of a diazonium salt. Their method is not entirely satisfactory because of diffusion artifacts which make fine localization impossible,[115] although for gross orientation it produces usable results. By replacing β-naphthyl acetate with the corresponding ester of α-naphthol or of naphthol AS and by selecting the right pH and temperature, it is possible to avoid diffusion artifacts completely and to obtain exceedingly sharp pictures on a cytological scale.

1) *The α-naphthol technique.*—Although the optimal activity of esterase in case of phenolic substrates is around pH 6.5, it is advisable to run the incubation at a pH of ±8 because of the much prompter azo-coupling of α-naphthol at this pH. The loss in enzymatic activity at this nonoptimal pH is a decided advantage, especially in the case of highly active tissues, such as liver, intestine, pancreas, etc., which often liberate naphthol at such a fast rate that the supply of diazonium salt cannot keep pace with it (see p. 146). Even at pH 8, diffusion may be quite disturbing. It is readily recognizable, even grossly, by the development of off-shades in the more active tissues; if it does occur, the incubating mixture must be refrigerated and/or stirred.

114. Gomori, G.: *ibid.*, **67**:4, 1948.
115. Gomori, G.: J. Lab. & Clin. Med., **35**:802, 1950.

Method

Prepare a stock solution of 1 per cent α-naphthyl acetate (Eastman No. 2380) in 50 per cent acetone; it will keep in the refrigerator indefinitely. For use, blow 0.5–1 ml. of the stock solution into about 50 ml. of distilled water, add a few ml. of a 0.2 M solution of disodium phosphate and 20–50 mg. of a diazonium salt (preferably one of the following: Diazo Blue B, Diazo Fast Blue RR, diazotized α-naphthylamine, or Diazo Red RC); stir the mixture and filter it into a Coplin jar. Do not protect sections with collodion because it may be stained by the dye. Incubate slides at a temperature not over 20° C. for 5–20 minutes or until the desired intensity of shade is obtained. Rinse in water, counterstain as desired, and mount in glycerol-jelly or balsam, depending on the diazonium salt used (see p. 170).

The sensitivity of the α-naphthol method is around 40–50 μM hydrolyzed in 10 minutes per gram of active structure at 10° C. (by Coujard's method).

2) *The naphthol AS technique.*—The acetate of naphthol AS (the anilide of 2-hydroxy-3-naphthoic acid) is hydrolyzed about six times slower than the acetate of α-naphthol; this may prove of considerable advantage in the case of highly active tissues, because no precautions are required to prevent diffusion artifacts. On the other hand, it is much less soluble in water than α-naphthol acetate, and, to obtain a high enough concentration, propylene glycol must be added to the incubating mixture. Naphthol AS acetate is not available on the market; it must by synthesized by esterifying naphthol AS with acetic anhydride in pyridine. It forms white crystals, melting at 159° ± 0°5 C. The previously published[116] melting point of 161° C. is erroneous.

Method

Dissolve 1 per cent of naphthol AS acetate in a mixture of equal parts of acetone and propylene glycol. Keep this stock

116. Gomori, G.: The histochemistry of esterases. In International review of cytology, Vol. 1. New York: Academic Press, 1952.

solution in the refrigerator. For use, blow 0.5–1 ml. of the stock solution into 10–15 ml. of propylene glycol. Shake the mixture. Dilute it, under continuous stirring, with water to 50 ml. Add a few ml. of 0.2 M phosphate buffer of pH ± 6.5. The mixture may be slightly opalescent. Add 20–50 mg. of Diazo Garnet GBC Salt; stir, and filter the solution into a Coplin jar. Incubate slides at room temperature for 30 minutes to 2 hours or until the desired intensity of shade is obtained. Rinse sections, counterstain with hematoxylin, differentiate thoroughly in acid alcohol, wash under the tap, and mount in glycerol-jelly.

The sensitivity of the naphthol AS method is around 30 μM in 1 hour per gram of active structure, in terms of α-naphthol (by Coujard's method).

3) *Methods utilizing other naphthol esters.*—As mentioned, β-naphthyl acetate should not be used if accurate localization is important. However, if, for special reasons, this method is chosen, it can be used exactly as described under the α-naphthol method. The pH of the incubating mixture should be 8–8.3; refrigeration and stirring will be found necessary in most cases. The best diazonium salts to be used with β-naphthol are Garnet GBC and diazotized α-naphthylamine. The acetate of 6-bromo-β-naphthol and the benzoates of α-naphthol and naphthol AS are very slow substrates, requiring an incubation of several hours; their use is not recommended.

Barrett and Seligman[117] suggest indoxyl acetate as a substrate; the indoxyl liberated is demonstrated as indigo. An objection to this type of a method has been voiced on page 147.

II. CHOLINESTERASES

Choline esters used in physiological and pharmacological experiments (acetate, butyrate, benzoate, etc.) are not suitable for histochemical use, because no reagents are known to precipitate the products of hydrolysis. This difficulty can be circumvented by (1) using long-chained fatty acid esters of

117. Barrett, R. J., and Seligman, A. M.: Science, **114**:579, 1951.

choline and demonstrating the acid moiety as in the Tween method and (2) using esters of thiocholine and precipitating free thiocholine as its cupric salt.

A. *The long-chained fatty acid ester technique.*—

Esters of choline with higher fatty acids are hydrolyzed by some cholinesterases at rates very much slower than acetylcholine[118] but still high enough to be useful for histochemical purposes.

The chlorides of lauroyl-, myristoyl-, palmitoyl- and stearoylcholine can be synthesized with ease. Myristoylcholine is available commercially.[119] All these esters are white crystalline solids, fairly soluble in distilled water. Pamitoyl and stearoylcholine will precipitate in the presence of salts unless some propylene glycol is added to the mixture. Stearoylcholine is hydrolyzed so slowly that its use is not recommended.

The principle of the technique[120] is identical with that of the Tween method for esterase-lipase. However, the technical details had to be modified because of the necessity of using relatively short-chained substrates (C_{12}–C_{16}). The Ca soaps of the shorter fatty acids are very coarsely crystalline, and for this reason Ca is replaced by Co, the corresponding salts of which are relatively fine granular precipitates. (Co is not recommended for use in the Tween technique because it markedly inhibits esterase-lipase.) The best buffer is tris-maleate, which prevents the impregnation of the tissue by Co, which otherwise may produce an unpleasant darkish background.

Method

Fixation as in the case of esterases. Collodion protection of the slides is advisable.

1. Prepare a 0.02 M ($\pm$ 0.7 per cent) stock solution of the

118. Glick, D.: J. Biol. Chem., **137**:357, 1941.
119. From Dajac Laboratories, 511 Lancaster Ave., Leominster, Mass.
120. Gomori, G.: Proc. Soc. Exper. Biol. & Med., **68**:354, 1948.

substrates in distilled water; keep them in the icebox. The solutions are more or less opalescent and quite viscous.

2. Prepare a buffered Co acetate solution containing about 0.0125 M Co acetate in a 0.025–0.03 M tris-maleate buffer of pH 7.6–7.8. Add about 0.5 ml. of 0.2 M solutions of each of the following salts to 100 ml. of the mixture: $MgCl_2$, $MnCl_2$, and $CaCl_2$ (activators). Add a few crystals of camphor.

For use, blow 1 ml. of the lauroyl- or myristoylcholine stock solution into 50 ml. of the buffered Co solution. In the case of palmitoylcholine, dissolve 1 ml. of the stock solution in 10–15 ml. of propylene glycol and dilute it with the Co solution to 50 ml. Filter the mixtures.

Incubate slides for 6–48 hours (lauroylcholine, 6–12 hours; myristoylcholine, 12–24 hours; palmitoylcholine, 24–48 hours) at 37° C. Wash slides under the tap. Immerse them in a dilute solution of yellow ammonium sulfide made up with 70 per cent alcohol instead of water to hasten the development of color. Counterstain as desired. Dehydrate and mount. Sites of activity are dark gray to black. With lauroylcholine the precipitate may be rather coarsely granular.

B. *The thiocholine technique.*[121]—

Esters of thiocholine are hydrolyzed by cholinesterases at about the same rates as, or faster than, the corresponding choline analogues. However, the specificity of the acetate for the "true" type of the enzyme is not so strict as that of acetylcholine.[122] Both cholinesterases will attack acetylthiocholine; butyrylthiocholine is attacked by the nonspecific type only. The nonspecific enzyme, regardless of the substrate used, is almost completely inhibited by 30 minutes' preincubation with 10^{-6} M of diisopropylfluorophosphate (DIPFP), whereas the "true" enzyme is inhibited only to the extent of 40 per cent. Any reaction obtained with butyrylthiocholine is due to the "pseudo"-enzyme only, while

121. Koelle, G. B., Friedenwald, J. S.: Proc. Soc. Exper. Biol. & Med., **70**:617, 1949.

122. Koelle, G. B.: J. Pharmacol & Exper. Therap., **100**:158, 1950.

any residual activity toward acetylthiocholine after pretreatment with DIPFP must be attributed to the "true" enzyme. Actually, in view of certain discrepancies, it is questionable whether this simple scheme can be accepted at face value. The hydrolysis of acetylthiocholine by liver is inhibited by DIPFP only to the extent of 70 per cent; this is contrary to the general consensus and the authors' own statement that the hepatic enzyme is exclusively of the nonspecific type.

Thiocholine, its acetate, and butyrate are available commercially.[123]

The method recommended is a simplification of the amended technique.[124]

Method

Make up the following stock solution:

Copper sulfate ($CuSO_4 \cdot 5H_2O$)	0.3 g.
Glycine	0.375 g.
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	1.0 g.
Maleic acid	1.75 g.
N (4 per cent) NaOH	30 ml.
Hot saturated (about 40 per cent) solution of Na_2SO_4	170 ml.

This solution will keep indefinitely, although some of the Na_2SO_4 may crystallize out on cooling. Its pH is around 6.

For use, dissolve about 20 mg. of acetylthiocholine iodide in few drops of water and add 10 ml. of the stock solution. Incubate teased tissues or fresh-frozen sections for 10–60 minutes at 37° C. Rinse them in two or three changes of saturated Na_2SO_4 and immerse them in a dilute solution of yellow ammonium sulfide. Counterstain as desired, dehydrate, and mount. Sites of activity are stained dark brown (shade of cupric sulfide).

If DIPFP is used as an inhibitor, the tissues are first treated with a 10^{-6} M solution of the compound in saturated

123. From LaWall and Harrison, 1921 Walnut St., Philadelphia 3, Pa.
124. Koelle, G. B.: J. Pharmacol. & Exper. Therap., **103**:153, 1951.

Na_2SO_4 for 30 minutes and then incubated with the substrate containing the same concentration of DIPFP.

Seligman and co-workers[125] have synthesized three highly interesting naphtholic substances which closely imitate the steric configuration of acetylcholine. No reports have been published so far on their practical use.

The Results of Histochemical Methods for Esterases

Saturated Tweens are hydrolyzed by many tissues; unsaturated ones, only by the pancreas of all species and by the chief cells of the mouse stomach ("true lipase").

The localization of enzymatic activity in the case of naphtholic substrates is very smiliar to that seen with the Tween technique, except that it is more widespread. A number of sites entirely negative with Tweens stain very intensely with the azo dye method (renal tubules of man; ganglion cells in several species, etc.). On the other hand, there are a few structures which are Tween-positive but naphthol-negative (spermatic elements of the testis and chief cells of the stomach of the mouse). Although at most sites the two naphtholic substrates produce identical pictures, there are several examples of true substrate specificity directed toward α-naphthyl acetate or naphthol AS acetate, respectively. The villi of the duodenum of the rat, certain unidentified cells in the heart of the rat, the septal cells of the mouse lung, etc., stain very intensely with α-naphthyl acetate and faintly, or not at all, with naphthol AS acetate. The reverse holds true for Brunner's glands of the rat, for motor cells of the rabbit and rat, and for human mast cells. The differences are so clear-cut that it is safe to assume the existence of two individual enzymes (conveniently called "α-esterase" and "AS-esterase," respectively), characterized by the substrate specificities indicated. The pictures obtained with β-naphthol appear to be identical with those seen in the α-naphthol method; this

125. Seligman, A. M., Nachlas, M. M., Manheimer, L. H., Friedman, O. M., and Wolf, G.: Ann. Surg., **130**:333, 1949; Ravin, H. A., Tsou, Kwan-Chung, and Seligman, A. M.: J. Biol. Chem., **191**:843, 1951.

is rather surprising because naphthol AS is a derivative of β-naphthol. Benzoates as substrates give pictures indistinguishable from those produced by the acetates. There is no histochemical support for the existence of a special acetylesterase.[126]

Most of the localizations of cholinesterases are entirely different from those of the aliesterases, although at some sites the localizations coincide (livers of some species; pancreas of the dog). In the sympathetic ganglia, esterase is found usually (but not always) in the bodies of the ganglion cells themselves, whereas cholinesterase is found in the aborizations around them. 10^{-5} M eserine completely abolishes all activity if lauroyl- or myristoylcholine are used as substrates. In the case of palmitoylcholine, inhibition is only partial. This difference may be due to the fact that the inhibitor undergoes considerable decomposition on prolonged incubation.

With regard to the long-chained fatty acid ester method for cholinesterase, two important points must be mentioned:

1. There are marked species and organ differences in respect to preference for the various substrates. Although, as a rule, lauroylcholine is hydrolyzed much faster than palmitoylcholine, rat and pigeon tissues and human adrenal medulla split palmitoylcholine as fast as, or faster than, lauroylcholine. The spermatic elements of the mouse testis show no reaction with lauroylcholine, a moderate reaction with myristoylcholine, and an intense one with palmitoylcholine, although other tissues of the mouse behave in the opposite way. The pattern of fine localization is also different with the three substrates. With lauroylcholine it is the nuclei which show the highest activity; with palmitoylcholine, cytoplasmic structures. These findings would indicate that the cholinesterases demonstrable by this method form a family

126. Jansen, E. F., Jang, R., and MacDonnell, L. R.: Arch. Biochem., **15**:415, 1947; Jansen, E. F., Nutting, M. D. F., and Balls, A. K.: J. Biol. Chem., **175**:975, 1948.

of enzymes with somewhat divergent, but overlapping, substrate specificities.

2. The biochemical difference between "true" and "pseudo"-cholinesterase does not hold histochemically. Some of the "true" cholinesterases (brain and muscle spindles of the mouse, brain of the dog, etc.) do split the long-chained fatty acid esters, others (brain of man and the rat and of all species phylogenetically below birds) do not. The same sort of situation applies to the "pseudo"-cholinesterases. The ability or inability to hydrolyze these esters is a feature which bisects the class of cholinesterases in a plane different from that which divides them into the "true" and the "pseudo" types. The two classifications have no common basis for comparison.

The thiocholine method appears to give results in better harmony with biochemical findings. At the present time, however, the material studied and reported is too scanty to permit any meaningful comparison of the activity patterns with those of the other methods. It is for this reason that the results of the thiocholine method will not be included in the following attempt at the histochemical classification of esterases.

After a careful analysis of hundreds of slides containing many tissues of a number of species, stained for the various esterases, the writer came to the conclusion that animal tissues contain three cardinal types of esterases, with narrowly defined specificities, and, in addition, a number of intermediate types which hydrolyze the substrates of two, or even of all three, of the main types. Whether the intermediate types actually represent individual enzymes with transitional properties or only the topographic compresence of several of the main types cannot be decided on the basis of data available. Table 6 gives a tentative classification of esterases demonstrable in paraffin sections by the Tween, azo dye, and the long-chained fatty acid choline ester techniques.

TABLE 6

The Histochemical Classification of Esterases

Enzymes	Substrates					Examples
	Saturated Tweens	Unsaturated Tweens	α-Naphthyl Acetate	Naphthol AS Acetate	Choline Esters	
I. Main types:						
A) Lipase	+	+	−	−	−	Chief cells of mouse stomach
B) Esterase	−	−	+	+	−	Chief cells of rabbit and dog stomach; human renal tubules; sympathetic ganglia in several species
B_1) α-esterase	−	−	+	− to ±	−	Unidentified cells in the rat heart; ampullary gland of the mouse
B_2) AS esterase	−	−	− to ±	+	−	Brunner's glands of the rat; cells of pancreatic islets of rat; motor cells of rabbit and rat; astroglia of cat
C) Cholinesterase	−	−	−	−	+	Human adrenal medulla; certain cells and tracts of mouse brain; arborizations around sympathetic ganglion cells in several species
II. Intermediate types:						
A–B	+	− +*	+	+	−	Chief cells of human stomach; pancreas of rat and mouse (also hydrolyze unsaturated Tweens); bronchial epithelium in several species; interstitial cells of rat testis
$A–B_1$	+	−	+	−	−	Duodenal villi of the rat; septal cells of mouse lung
$A–B_2$	+	−	−	+	−	No examples known
A–C	+	−	−	−	+	Spermatic elements of mouse testis
B–C	−	−	+	+	+	Muscle spindles of mouse; conductive system of dog's heart; Bowman's capsules in dog's kidney
$B_1–C$	−	−	+	−	+	No examples known
$B_2–C$	−	−	−	+	+	No examples known
A–B–C	+	− +*	+	+	+	Pancreas of several species (also hydrolyzes unsaturated Tweens); liver and intestine of several species

* Pancreas only.

β-Glucuronidase

The enzyme β-glucuronidase seems to play an important role in detoxication mechanisms, in the metabolism of phenolic steroids,[127] and in the urinary excretion of phenolic compounds. Phenol, camphor, and phenolic steroids are excreted, at least in part, as glucuronides. Another interest of the enzyme lies in the fact that malignant tissues often contain fairly high concentrations of it.[128]

Regular chemical synthesis of glucuronides is a very difficult task; for this reason the substrates are usually prepared by biosynthesis. Rabbits are given phenolic compounds by mouth or intravenously, and the glucuronide is isolated from the urine.

The histochemical demonstration of glucuronidase is fraught with difficulties. First of all, even the most active tissues contain very little glucuronidase as compared to other enzymes. A glucuronidase activity of 50 μM of substrate hydrolyzed per gram of tissue per hour is exceptional, whereas the alkaline phosphatase activity of the rat kidney is around 1,000 μM, and the esterase activity of rat liver 4,000–6,000 μM. Secondly, the enzyme is quite sensitive to fixatives, and this fact makes the use of fresh tissues almost imperative. However, according to recent data of Seligman and co-workers,[129] fixation in formalin should be possible. Thirdly, the pH optimum of the enzyme is in the acid range, unfavorable for azo-coupling. Friedenwald and Becker[130]

127. Fishman, W. H., and Fishman, L. W.: J. Biol. Chem., **152**:487, 1944; Fishman, W. H.: J. Biol. Chem., **169**:7, 1947.

128. Fishman, W. H.: Science, **105**:646, 1947; Fishman, W. H., and Anlyan, A. J.: Science, **106**:66, 1947; Fishman, W. H., and Anlyan, A. J.: Cancer Research, **7**:808, 1947; Fishman, W. H., and Anlyan, A. J.: J. Biol. Chem., **169**:449, 1947; Odell, L. D., and Burt, J. C.: Cancer Research, **9**:362, 1949; Odell, L. D., Burt, J. C., and Bethea, R.: Science, **109**:564, 1949.

129. Seligman, A. M., Chauncey, H. H., and Nachlas, M. M.: Stain Technol., **26**:19, 1951.

130. Friedenwald, J. S., and Becker, B.: J. Cell. & Comp. Physiol., **31**:303, 1948.

devised two methods to avoid the latter difficulty: (1) 8-hydroxyquinoline glucuronide is used as a substrate, and 8-hydroxyquinoline is precipitated as its ferric salt; (2) *o*-aminophenol glucuronide is prepared first by biosynthesis, then diazotized, and coupled with β-naphthol. In this way the glucuronide of β-naphthylazo-o-phenol is obtained. This is a red dye, slightly soluble in water, while the phenol itself, liberated from it by enzymatic action, is insoluble and will precipitate. Seligman and co-workers[131] object to the use of a colored substrate, fearing nonenzymatic staining effects. They propose the use of glucuronides of highly insoluble naphthols, such as 6-bromo-β-naphthol or 8-benzenesulfonamido-β-naphthol; incubation is to be carried out in the absence of a diazonium salt, and azo-coupling of the precipitated naphthol is performed in a second step. No reports have been published so far on the actual use of this substrate. As pointed out before (p. 171), this type of technique is not likely to work out satisfactorily.

Since the substrates are not available commercially and are almost impossible for most workers in the biological fields to prepare, only the essential features of the Friedenwald-Becker methods will be given here.

Fresh-frozen sections are used; the substrate is buffered with acetate at pH 5.

1. Incubate tissues in a saturated solution of β-naphthylazo-*o*-phenyl glucuronide for 1–5 hours. Wash sections, fix them in formalin, and counterstain with hematoxylin. Mount in glycerol jelly. Sites of activity are brilliant red; background, orange-yellow.

2. Saturate a solution of 8-hydroxyquinoline glucuronide containing 0.03 N ferric chloride with ferric hydroxyquinoline. This saturation of the incubating medium with the compound to be produced enzymatically is essential. Incu-

131. Seligman, A. M., Nachlas, M. M., Manheimer, L. H., Friedman, O. M., and Wolf, G.: Ann. Surg., **130**:333, 1949; Seligman, A. M., and Nachlas, M. M.: Cancer Research, **10**:240, 1950.

bate sections for 3–18 hours. Remove excess Fe ions bound by tissue proteins with an oxalate buffer of pH 4. Convert ferric hydroxyquinoline into Prussian blue by treating the sections with an acidified solution of potassium ferrocyanide. Counterstain with a red nuclear stain. Sites of activity are blue.

Campbell[132] reports the successful use of these methods.

Carbonic Anhydrase

Kurata[133] has described a histochemical method for the localization of carbonic anhydrase, based on the following principle:

If a solution of a salt of a heavy metal (such as manganese or cobalt) is added to a solution of sodium bicarbonate, a precipitate of metal carbonate (and bicarbonate) will form. At the same time some carbon dioxide will be liberated, owing to the acid reaction of salts of heavy metals. This free carbon dioxide will lower the pH of the solution slightly. On standing, the pH will gradually shift back in the direction of the equilibrium value (± 8), and additional metal carbonate will precipitate. In the presence of carbonic anhydrase this reaction will be greatly accelerated.

Kurata fixes thin slices of tissue in cold acetone for 1 hour, washes them briefly in distilled water, and incubates them in a freshly prepared and filtered medium at 37° C. for 45 minutes. The medium is a mixture of 100 ml. of an 8 per cent solution of Na bicarbonate and of 10 ml. of a 10 per cent solution of either $MnCl_2$ or $CoCl_2$. The tissues are washed with a bicarbonate buffer of pH $\pm$ 7.2 and embedded in paraffin. The sections are treated with ammonium sulfide to reveal $CoCO_3$ as black CoS or with neutral periodate to convert $MnCO_3$ into dark-brown MnO_2. Typical localizations (parietal cells of the gastric mucosa; red cells) are claimed.

132. Campbell, J. G.: Brit. J. Exper. Path., **30**:548, 1949.
133. Kurata, K.: Tr. Soc. Path. Jap., **38**:108, 1949.

Urease

Sen[134] described a method for the demonstration of urease in the jack bean. The unfixed seeds are incubated with urea and cobalt chloride in 60–80 per cent alcohol (urease is active in an alcoholic medium). The precipitate of cobalt carbonate is demonstrated in the form of sulfide.

The writer finds that the method does not work with animal tissues.

APPENDIX

Buffers for Use in Histochemistry

The pH values in the tables which follow are only approximate but are sufficiently accurate for histochemical purposes.

0.2 M citrate buffer

Solution A: 4.2 g. of citric acid in 100 ml. of water.
Solution B: 5.9 g. of sodium citrate in 100 ml. of water.
Add a few crystals of camphor to both solutions.

A (ml.)	B (ml.)	pH	A (ml.)	B (ml.)	pH
80	20	3.4	55	45	4.5
76	24	3.6	46	54	4.8
70	30	3.8	40	60	5.0
65	35	4.0	35	65	5.3
61	39	4.2	30	70	5.5

0.2 M acetate buffer

Solution A: 1.2 ml. of acetic acid in 100 ml. of water.
Solution B: 2.7 g. of Na acetate ($CH_3COONa \cdot 3H_2O$) in 100 ml. of water.
Add a few crystals of camphor to both solutions.

A (ml.)	B (ml.)	pH	A (ml.)	B (ml.)	pH
87	13	3.8	40	60	4.8
80	20	4.0	30	70	5.0
73	27	4.2	21	79	5.2
62	38	4.4	14.5	85.5	5.4
51	49	4.6	11	89	5.6

134. Sen, P. B.: Indian J. M. Research, **18**:79, 1930.

0.2 M MALEATE BUFFER

50 ml. 0.4 M acid Na maleate (2.32 g. of maleic acid + 20 ml. of N [4 per cent] NaOH in 50 ml.) + x ml. of 4 per cent NaOH diluted to a total of 100 ml.

ml. NaOH	pH	ml. NaOH	pH
0.5	4.6	5.8	5.6
1.0	4.8	7.6	5.8
1.8	5.0	10.0	6.0
2.8	5.2	12.5	6.2
4.0	5.4	14.5	6.4

Add a few crystals of camphor.

0.2 M PHOSPHATE BUFFER

Solution A: monobasic Na phosphate ($NaH_2PO_4 \cdot H_2O$), 2.76 g. in 100 ml.

Solution B: dibasic Na phosphate ($Na_2HPO_4 \cdot 7H_2O$), 5.36 g., or $Na_2HPO_4 \cdot 12H_2O$, 7.6 g. in 100 ml.

A (ml.)	B (ml.)	pH	A (ml.)	B (ml.)	pH
90	10	5.9	33	67	7.1
85	15	6.1	23	77	7.3
77	23	6.3	19	81	7.4
68	32	6.5	16	84	7.5
57	43	6.7	10	90	7.7
45	55	6.9			

0.2 M TRIS(HYDROXYMETHYL)AMINOMETHANE-MALEATE-(TRIS-MALEATE) BUFFER

Dissolve 29 g. of maleic acid and 30.3 g. of tris(hydroxymethyl)-aminomethane (Commercial Solvents Corp., New York 17) in 500 ml. of distilled water. Add about 2 g. of charcoal, shake, let stand for 10 minutes, and filter. 40 ml. of this stock solution + x ml. of N (4 per cent) NaOH diluted to a total of 100 ml.

ml. NaOH	pH	ml. NaOH	pH	ml. NaOH	pH
9.0	5.8	16.5	6.6	22.5	7.6
10.5	6.0	18.0	6.8	24.2	7.8
13.0	6.2	19.0	7.0	26.0	8.0
15.0	6.4	20.0	7.2	29.0	8.2

Add a few crystals of camphor.

0.05 M BARBITAL BUFFER

1.03 g. of Na barbital in 50 ml. + x ml. of 0.1N HCl. diluted to a total volume of 100 ml.

HCl (ml.)	pH	HCl (ml.)	pH
5.0	8.7	26	7.65
7.5	8.5	31	7.45
11	8.3	36	7.3
15	8.1	41	7.15
19	7.9	43.5	6.9

Barbital buffers of higher molarity than 0.05 M should not be used because barbituric acid will precipitate, especially in the lower ranges of pH.

0.2 M 2-AMINO-2-METHYL-1,3-PROPANEDIOL BUFFER

2.1 g. substance (Commercial Solvents Corp., New York 17) in 50 ml. + x ml. of N HCl, diluted to a total of 100 ml.

HCl (ml.)	pH	HCl (ml.)	pH
2.0	9.6	11.0	8.6
3.0	9.4	13.5	8.4
5.0	9.2	15.0	8.2
7.0	9.0	16.0	8.0
9.0	8.8		

0.2 M BORATE BUFFER

Solution A: 1.24 g. of boric acid in 100 ml.
Solution B: 1.9 g. of borax ($Na_2B_4O_7 \cdot 10H_2O$) in 100 ml.

A (ml.)	B (ml.)	pH	A (ml.)	B (ml.)	pH
90	10	7.4	55	45	8.4
85	15	7.6	45	55	8.6
80	20	7.8	30	70	8.8
70	30	8.0	20	80	9.0
65	35	8.2	10	90	9.2

ADDITIONAL REFERENCES

ADDITIONAL REFERENCES

CHAPTER VI

Potassium

Gersh, I.: Anat. Rec., **70**:311, 1938.
Marza, V. D.: Bull. d'histol. appliq. à la physiol., **13**:62, 1936.
Marza, V. D., and Chiosa, L.: Bull. d'histol. appliq. à la physiol., **12**:58, 1935.
Marza, V. D., and Chiosa, L.: *Ibid.*, **13**:153, 1936.
Rohdenburg, G. L., and Geiger, J.: Arch. Path., **6**:215, 1928.

Calcium

Gersh, I.: Anat. Rec., **70**:331, 1938.
Okamoto, K.; Seno, M.; and Kato, A.: Taishitzu Gaku Zasshi, **13**:72, 1944.

Iron

Bunting, H.: Stain Technol. **24**:109, 1949.
Grynfeltt, E., and Cristol, P.: Bull. Soc. chim. biol. **5**:797, 1923.
Nishimura, J.: Zentralbl. f. allg. Path. u. path. Anat., **21**:10, 1910.
Stoeltzner, W.: Zentralbl. f. allg. Path. u. path. Anat., **30**:225, 1919.
Villaret, M.; Justin-Besançon, L.; Doubrow, S.; and Even, R.: Compt. rend. Soc. de biol., **108**:954, 1931.

Lead

Iwahashi, U.: Tr. Jap. Path. Soc., **16**:129, 1926.

Bismuth

Califano, L.: Ztschr. f. Krebsforsch., **26**:183, 1928.
Christeller, E.: Med. Klin., **22**:619, 1926.
Gutzeit, G., and Weibel, R.: Compt. rend. Soc. de phys. et hist. nat. Genève, **51**:33, 1934.
Okamoto, K.: Taishitzu Gaku Zasshi, **13**:58, 1944.

Silver

Okamoto, K.; Utamura, M.; and Akagi, T.: Acta Scholae Med. Univ. Kioto, **22**:361, 1938–39.

Gold

Okamoto, K.; Akagi, T.; and Mikami, G.: Acta Scholae Med. Univ. Kioto, **22**:373, 1938–39.

PALLADIUM

OKAMOTO, K.; MIKAMI, G.; and NISHIDA, M.: Acta Scholae Med. Univ. Kioto, **22**:382, 1938–39.

COBALT

OKAMOTO, K., and SONODA, H.: Taishitzu Gaku Zasshi, **13**:62, 1944.

ALUMINUM

OKAMOTO, K.: Taishitzu Gaku Zasshi, **13**:66, 1944.

TIN

OKAMOTO, K.: Taishitzu Gaku Zasshi, **13**:69, 1944.

URANIUM

SCHNEIDER, G.: Skandinav. Arch. f. Physiol., **14**:383, 1903.

CHLORIDE

LESCHKE, E.: Ztschr. f. klin. Med., **81**:14, 1915.
LISON, L.: Ztschr. f. Zellforsch. u. mikr. Anat., **25**:143, 1936.

CHAPTER VII

THEORETICAL AND TECHNICAL

ARZAC, J. P.: Analecta Med., **8**:9, 1947.
ARZAC, J. P., and FLORES, L. G.: Stain Technol., **24**:25, 1949.
BANK, O., and BUNGENBERG DE JONG, H. G.: Protoplasma, **32**:489, 1939.
HESS, M., and HOLLANDER, F.: J. Lab. & Clin. Med., **29**:321, 1944.
HIGHMAN, B.: Stain Technol., **20**:85, 1945.
LANDSMEER, J. M. F.: Acta physiol. & pharmacol. Neerland., **2**:112, 1951.
LILLIE, R. D.: J. Lab. & Clin. Med., **32**:76, 1947.
LILLIE, R. D.: *Ibid.*, **32**:910, 1947.
LILLIE, R. D.: J. Nat. Cancer Inst., **10**:1344, 1950.
LISON, L.: Acad. roy. Belgique, cl. de sc., **20**:1160, 1934.
LISON, L.: Protoplasma, **24**:453, 1935.
LISON, L.: Bull. Soc. chim. biol., **18**:225, 1936.
MCMANUS, J. F. A.: Stain Technol., **23**:99, 1948.
MCMANUS, J. F. A., and CASON, J. E.: J. Exper. Med., **91**:651, 1950.
MCMANUS, J. F. A., and CASON, J. E.: J. Nat. Cancer Inst., **10**:1343, 1950.
MANNOZZI TORINI, M.: Med. sper., arch. ital., **16**:57, 1942.

METZNER, R., and KRAUSE, R.: In Abderhalden's Handb. d. biol. Arbeitsmeth., Abt. 5, **2-1**:359, 1928.
SHABADASH, A. L.: Izvest. Akad. Nauk SSSR, **1**:745, 1947.
SPEK, J.: Protoplasma, **34**:533, 1940.

GLYCOGEN

BONDY, P. K., and SHELDON, W. H.: Proc. Soc. Exper. Biol. & Med., **65**:68, 1947.
EDLUND, Y., and HOLMGREN, H.: Ztschr. f. mikr.-anat. Forsch., **47**:467, 1940.
GENDRE, H.: Bull. d'histol. appliq. à la physiol., **14**:262, 1937.
GIBB, R. P., and STOWELL, R. E.: Blood, **4**:569, 1949.
GRAFFLIN, A. L.; MARBLE, A.; and SMITH, R. M.: Anat. Rec. **81**:495, 1941.
MCMANUS, J. F. A., and FINDLEY, L.: Surg., Gynec. & Obst., **89**:49, 1949.
MANCINI, R. E.: Anat. Rec., **101**:149, 1948.
MANCINI, R. E., and CELANI-BARRY, R.: Rev. Soc. argent. de biol., **17**:499, 1941.
PRATI, A.: Boll. Soc. med.-chir. Pavia, **20**:603, 1942.
WALLRAFF, J., and BECKERT, H.: Ztschr. f. mikr.-anat. Forsch., **45**:511, 1939.
WALLRAFF, J., and BEDNARA-SCHÖBER, M.: Ztschr. f. mikr.-anat. Forsch., **53**:102, 1943.
WISLOCKI, G. B.; RHEINGOLD, J. J.; and DEMPSEY, E. W.: Blood, **4**:562, 1949.

MUCIN AND CONNECTIVE-TISSUE POLYSACCHARIDES

BIGNARDI, C.: Boll. di zool., **10**:213, 1939.
BIGNARDI, C.: Atti Soc. nat. e mat. Modena, **71**:63, 1940.
BIGNARDI, C.: Boll. Soc. med.-chir. Pavia, **54**:803, 1940.
BIGNARDI, C., and CASELLA, C.: Boll. di zool., **12**:83, 1941.
CATCHPOLE, H. R.: Ann. New York Acad. Sc., **52**:989, 1950.
DEMPSEY, E. W.; BUNTING, H.; SINGER, M.; and WISLOCKI, G. B.: Anat. Rec., **98**:417, 1947.
EWER, D. W., and HANSON, J.: J. Roy. Micr. Soc., **65**:40, 1945.
FOLLIS, R. H.: J. Nat. Cancer Inst., **10**:1370, 1950.
JÄRVI, O., and TEIR, H.: Acta Soc. med. Fenn. "Duodecim," A, **23**:7, 1947.
KULONEN, E.: Acta path. et microbiol. Scandinav., **27**:461, 1950.
LEBLOND, C. P.; CLERMONT, Y.; and CIMON, L.: Anat. Rec., **106**:306, 1950.
ROMANINI, M. G.: Acta anat., **13**:256, 1951.

Sylvén, B.: Virchows Arch. f. path. Anat., **303**:280, 1939.
Wislocki, G. B.: J. Nat. Cancer Inst., **10**:1341, 1950.
Wislocki, G. B.; Bunting, H.; and Dempsey, E. W.: Am. J. Anat., **81**:1, 1947.

Heparin, Mast Cells

Ehrström, M. C.: Acta med. Scandinav., **101**:551, 1939.
Friberg, U.; Graf, W.; and Aberg, B.: Acta path. et microbiol. Scandinav., **29**:197, 1951.
Jorpes, E.: Biochem. J., **29**:1817, 1935.
Jorpes, E.: Naturwiss., **23**:196, 1935.
Jorpes, E.; Werner, B.; and Åberg, B.: J. Biol. Chem., **176**:277, 1948.
Sylvén, B.: Acta chir. Scandinav., **86**, Suppl. 66, 1941.
Sylvén, B.: J. Bone & Joint Surg., **29**:973, 1947.

Gonadotropic Hormone

Catchpole, H. R.: Fed. Proc., **6**:88, 1947.
Herlant, M.: Arch. de biol., **54**:225, 1942–43.
Herlant, M.: Rev. canad. de biol., **9**:113, 1950.
Pearse, A. G. Everson: Nature, **162**:651, 1948.
Pearse, A. G. Everson: J. Path. & Bact., **61**:195, 1949.
Pearse, A. G. Everson: Stain Technol., **25**:95, 1950.
Pearse, A. G. Everson, and Rinaldini, L. M.: Brit. J. Exper. Path., **31**:540, 1950.

Other Polysaccharides

Gersh, I.: Fed. Proc., **6**:392, 1947.
McManus, J. F. A.; Saunders, J. C.; Penton, G.B.; and Cason, J. E.: Science, **111**:155, 1950.
Monné, L., and Slautterback, D. B.: Exper. Cell Research, **1**:477, 1950.

Enzyme Studies

Follis, R. H., Jr., and Berthrong, M.: Bull. Johns Hopkins Hosp., **85**:281, 1949.
Lillie, R. D.: Anat. Rec., **103**:611, 1949.
Lillie, R. D.; Greco, J.; and Laskey, A.: Anat. Rec., **103**:635, 1949.

Nucleic Acids

general reviews

Davidson, J. N.: The biochemistry of the nucleic acids. London: Methuen & Co., 1950.

Lumb, E. S.: Quart. Rev. Biol., **25**:278, 1950.
Schultz, J.: Cold Spring Harbor Symp. Quant. Biol., **9**:55, 1941.

Validity of the Feulgen Technique

Brachet, J.: Symp. Soc. Exper. Biol., **1**:207, 1947.
Callan, H. G.: Nature, **152**:503, 1943.
Di Stefano, H. S.: Proc Nat. Acad. Sc., **34**:75, 1948.
Stacey, M.; Deriaz, R. E.; Teece, E. G.; and Wiggins, L. F.: Nature, **167**:740, 1946.
Stedman, E., and Stedman, E.: Nature, **152**:267, 1943.
Stowell, R. E.: Stain Technol., **20**:45, 1945.
Stowell, R. E.: *Ibid.*, **21**:137, 1946.

Quantitative Studies

Gersh, I., and Bodian, D.: J. Cell. & Comp. Physiol., **21**:253, 1943.
Naora, H.: Science, **114**:279, 1951.
Pollister, A. W., and Ris, H.: Cold Spring Harbor Symp. Quant. Biol., **12**:147, 1947.
Ris, H., and Mirsky, A. E.: J. Gen. Physiol., **33**:125, 1949.
Stowell, R. E.: J. Nat. Cancer Inst., **3**:111, 1942.
Swift, H. H.: Physiol. Zoöl., **23**:169, 1950.
Swift, H. H.: Proc. Nat. Acad. Sc., **36**:643, 1950.
Widström, G.: Biochem. Ztschr. **199**:298, 1928.

Enzyme Studies

Cooper, E. J.; Trautmann, M. L.; and Laskowski, M.: Proc. Soc. Exper. Biol. & Med., **73**:219, 1950.
Fischer, F. G.; Böttger, I.; and Lehmann-Echternacht, H.: Ztschr. f. physiol. Chem., **271**:246, 1941.
Kunitz, M.: Science, **90**:112, 1939.
Kunitz, M.: J. Gen. Physiol., **24**:15, 1940.
Lamanna, C., and Mallette, M. F.: Arch. Biochem., **24**:451, 1949.
McDonald, M. R.: J. Gen. Physiol., **32**:39, 1948.
Mazia, D.: Cold Spring Harbor Symp. Quant. Biol., **9**:40, 1941.
Mazia, D., and Jaeger, L.: Proc. Nat. Acad. Sc., **25**:456, 1939.
Sanders, F. K.: Quart. J. Micr. Sc. **87**:203, 1946.
Stowell, R.E., and Zorzoli, A.: Stain Technol., **22**:51, 1947.
Vincent, W. S., and Dornfeld, E. J.: Am. J. Anat., **83**:437, 1948.

Nucleic Acids in Experimental and Pathological Conditions

Crouse, H. V.; Coriell, L. L.; Blank, H.; and McNair, S. T. F.: J. Nat. Cancer Inst., **10**:1372, 1950.

Gersh, I., and Bodian, D.: Biol. Symp., **10**:163, 1943.
Gössner, W.: Zentralbl. f. allg. Path. u. path. Anat., **85**:434, 1949.
Klemperer, P.; Gueft, B.; Lee, S. L.; Leuchtenberger, C.; and Pollister, A. W.: Arch. Path., **49**:503, 1950.
Sulkin, N. M.: Anat. Rec., **106**:290, 1950.

NUCLEIC ACIDS IN BACTERIA

DeLamater, E. D.: Stain Technol., **26**:199, 1951.
Dubos, R. J.: The bacterial cell, pp. 72 and 354. Cambridge, Mass.: Harvard University Press, 1945.
Piekarski, G.: Ergebn. d. Hyg., **26**:333, 1949.
Vendrely, R., and Lipardy, J.: Compt. rend. Acad. sc., **223**:343, 1946.

APPENDIX

Bourne, G.: Nature, **131**:874, 1933.
Bourne, G.: Australian J. Exper. Biol., **13**:113, 1935.
Bourne, G.: *Ibid.*, **13**:239, 1935.
Bourne, G.: Nature, **153**:255, 1944.
Bourne, G.: *Ibid.*, **166**:549, 1950.
Day, M. F.: Australian J. Sc. Research, ser. B, Biol. Sciences, **2**:19, 1949.
Galvão, P. E., and Cardoso, D. M.: Compt. rend. Soc. de biol., **115**:350, 1934.
Giroud, A., and Leblond, C. P.: Arch. d'anat. micr., **31**:111, 1935.
Giroud, A., and Leblond, C. P.: L'Acide ascorbique dans les tissus et sa détection. Paris: Hermann & Cie, 1936.
Gough, J., and Zilva, S. S.: Biochem. J., **27**:1279, 1933.
Tonutti, E.: Protoplasma, **31**:151, 1938.

Lipids

GENERAL TECHNIQUE

Lison, L.: Bull. d'histol. appliq. à la physiol., **10**:293, 1933.

OSMIUM REACTION

Hoerr, N. L.: Anat. Rec., **66**:149, 1936.

CHOLESTEROL

Larson, H. W.: J. Lab. & Clin. Med., **18**:848, 1933.
Okamoto, K.; Shimamoto, H.; and Sonoda, H.: Taishitzu Gaku Zasshi, **13**:113, 1944.
Romieu, M.: Compt. rend. Soc. de biol., **92**:787, 1925.
Rosenheim, O.: Biochem. J., **33**:47, 1929.

GRANULES OF LEUCOCYTES

Bruijn, P. P. H. de: Acta néerland. morph. norm. et path., **2**:232, 1939.
Discombe, G.: J. Path. & Bact., **58**:572, 1946.
McManus, J. F. A.: Nature, **156**:173, 1945.
Ruyter, J. H. C.: Ztschr. f. Zellforsch. u. mikr. Anat., **19**:106, 1933.
Sheehan, H. L.: J. Path. & Bact., **49**:580, 1939.
Sheehan, H. L., and Storey, G. W.: J. Path. & Bact., **59**:336, 1947.

VITAMIN A

Cornbleet, T., and Popper, H.: Arch. Dermat. & Syph., **46**:59, 1942.
Greenberg, R., and Popper, H.: Am. J. Physiol., **134**:114, 1941.
Jancsó, N. von, and Jancsó, H. von: Biochem. Ztschr., **287**:289, 1936.
Kado, H., and Nagai, T.: Tr. Soc. Path. Jap., **32**:105, 1942.
Meyer, K. A.; Popper, H.; Steigmann, F.; Walters, W. H.; and Zevin, S.: Proc. Soc. Exper. Biol. & Med., **49**:589, 1942.
Popper, H.: J. Mt. Sinai Hosp., **7**:119, 1940.
Popper, H.: Physiol. Rev., **24**:205, 1944.
Popper, H., and Brenner, S.: J. Nutrition, **23**:431, 1942.
Popper, H., and Greenberg, R.: Arch. Path., **32**:11, 1941.
Popper, H., and Ragins, A. B.: Arch. Path., **32**:258, 1941.
Popper, H.; Steigmann, F.; and Dyniewicz, H. A.: Proc. Soc. Exper. Biol. & Med., **50**:266, 1942.
Querner, F. R. von: Klin. Wchnschr., **14**:1213, 1935.
Ragins, A. B., and Popper, H.: Arch. Path., **34**:647, 1942.

LIPID ALDEHYDES

Barker, W. L.: Endocrinology, **48**:772, 1951.
Benua, R. S., and Howard, E.: Bull. Johns Hopkins Hosp., **86**:200, 1950.
Bergner, G. E., and Deane, H. W.: Endocrinology, **43**:240, 1948.
Cain, A. J.: Quart. J. Micr. Sc., **90**:411, 1949.
Deane, H. W., and Greep, R. O.: Am. J. Anat., **79**:117, 1946.
Deane, H. W., and Greep, R. O.: Endocrinology, **41**:243, 1947.
Deane, H. W.; Shaw, J. H.; and Greep, R. O.: Endocrinology, **43**:133, 1948.
Dempsey, E. W.: Ann. Rev. Physiol., **8**:451, 1946.
Dempsey, E. W., and Wislocki, G. B.: Physiol. Rev., **26**:1, 1946.
Guyon, L.: Compt. rend. Soc. de biol., **109**:1101, 1932.
McKay, D. G., and Robinson, D.: Endocrinology, **41**:378, 1947.
Motta, G.: Folia gynaec., **38**:519, 1931.

Oster, K. A.: Endocrinology, **36**:92, 1945.
Oster, K. A.: *Ibid.*, **37**:69, 1945.
Pollock, W. F.: Anat. Rec., **84**:23, 1942.
Rogers, W. F., and Williams, R. H.: Arch. Path., **44**:126, 1947.
Van Dorp, A. W. V., and Deane, H. W.: Anat. Rec., **107**:265, 1950.
Verne, J.: Bull. d'histol. appliq. à la physiol., **5**:223, 1928.
Verne, J.: Compt. rend. A. anat., **23**:465, 1928.
Verne, J.: Compt. rend. Soc. de biol., **99**:266, 1928.
Verne, J.: Rev. neurol., **1**:722, 1928.
Verne, J.: Arch. d'anat. micr., **25**:137, 1929.
Verne, J.: Compt. rend. Soc. de biol., **121**:609, 1936.
Verne, J.: Compt. rend. A. anat., **32**:440, 1937.
Wislocki, G. B.: Endocrinology, **44**:167, 1949.

Urea

Oestreicher, A.: Virchows Arch. f. path. Anat., **257**:614, 1925.
Stübel, H.: Anat. Anz., **54**:236, 1921.

Enterochromaffin Substance

Clara, M.: Ztschr. f. mikr.-anat. Forsch., **30**:467, 1932.
Clara, M.: Ergebn. d. Anat. u. Entwcklngsgesch., **30**:240, 1933.
Clara, M.: Ztschr. f. Zellforsch. u. mikr. Anat., **18**:435, 1933.
Hamperl, H.: Ztschr. f. mikr.-anat. Forsch., **2**:506, 1925.
Vialli, M.: Boll. Soc. ital. biol., **9**:203, 1934.
Vialli, M., and Erspamer, V.: Ztschr. f. Zellforsch. u. mikr. Anat., **27**:81, 1937–38.

Pigments

pigments in general

Bargmann, E.: Ergebn. d. Physiol., **35**:158, 1933.
Hueck, W.: Beitr. z. path. Anat. u.z. allg. Path., **54**:68, 1912.
Verne, J.: Les Pigments dans l'organisme animal. Paris: Doin, 1926.

ceroid

Brenner, S.: South African J. M. Sc., **11**:173, 1946.
Edwards, J. E., and Dalton, A. J.: J. Nat. Cancer Inst., **3**:19, 1942.
Edwards, J. E., and White, J.: J. Nat. Cancer Inst., **2**:157, 1941.
Hartroft, W. S.: Science, **113**:673, 1951.
Mason, K. E., and Emmel, A. F.: Anat. Rec., **92**:33, 1945.
Mason, K. E., and Emmel, A. F.: *Ibid.*, **92**:49, 1945.
Pappenheimer, A. M., and Victor, J.: Am. J. Path., **22**:395, 1946.
Wolf, A., and Pappenheimer, A. M.: J. Neuropath. & Exper. Neurol., **4**:402, 1945.

Zorzoli, G.: Boll. Soc. ital. biol. sper., **26**:138, 1950.
Zorzoli, G., and Felci, U.: Boll. Soc. ital. biol. sper., **26**:142, 1950.
Zorzoli, G., and Veneroni, G.: Boll. Soc. ital. biol. sper., **26**:140, 1950.

CAROTENOIDS

Escher, H. H.: Ztschr. f. physiol. Chem., **83**:198, 1913.
Schmidt, W. J.: Arch. f. mikr. Anat., **90**:98, 1917.
Verne, J.: Essai histochimique sur les pigments tégumentaires des crustacés décapodes. Paris: Doin, 1923 (No. 16, Arch. de morph. gén. et expér.).

CHAPTER VIII

Dehydrogenases

TELLURITE

Conradi, H., and Troch, P.: München. med. Wchnschr., **59**:1652, 1912.
Hasegawa, K.: Jap. J. Bot., **8**:1, 1936.
Wachstein, M.: Proc. Soc. Exper. Biol. & Med., **72**:175, 1949.

TRIPHENYLTETRAZOLIUM

Black, M. M.; Kleiner, I. S.; and Speer, F. D.: Cancer Research, **10**:204, 1950.
Black, M. M.; Speer, F. D.; and Opler, S. R.: Am. J. Path., **26**:741, 1950.
Mackenzie, L. L., and Fuller, D.: J. Lab. & Clin. Med., **35**:314, 1950.
Mattson, A. M.; Jensen, C. O.; and Dutcher, R. A.: Science, **106**:294, 1947.
Pratt, R., and Dufrenoy, J.: Stain Technol., **23**:137, 1948.
Roberts, L. W.: Science, **113**:692, 1951.
Straus, F. H.; Cheronis, N. D.; and Straus, E.: Science, **108**:113, 1948.
Zweifach, B. W.; Black, M. M.; and Shorr, E.: Proc. Soc. Exper. Biol. & Med., **76**:446, 1951.

NEOTETRAZOLIUM

Antopol, W.; Glaubach, S.; and Goldman, L.: Pub. Health Rep., **63**:1231, 1948.
Wachstein, M.: Proc. Soc. Exper. Biol. & Med., **72**:175, 1949.
Wachstein, M.: *Ibid.*, **73**:306, 1950.

DITETRAZOLIUM

Rutenburg, A. M.; Gofstein, R.; and Seligman, A. M.: Cancer Research, **10**:113, 1950.

Indophenol Oxidase

Fiessinger, N., and Rudowska, L.: Arch. de méd. expér., **24**:585, 1912.
Fursenko, B.: Zentralbl. f. allg. Path. u. path. Anat., **22**:97, 1911.
Gierke, E. von: Zentralbl. f. allg. Path. u. path. Anat., **27**:318, 1916.
Graeff, S.: Frankfurt. Ztschr. f. Path., **11**:358, 1912.
Graeff, S.: Zentralbl. f. allg. Path. u. path. Anat., **27**:313, 1916.
Graeff, S.: *Ibid.*, **35**:481, 1925.
Graeff, S.: Die mikromorphologischen Methoden der Fermentforschung im tierischen und pflanzlichen Organismus. In Abderhalden's Handb. d. biol. Arbeitsmeth., IV-**1**:93. Berlin and Vienna: Urban & Schwarzenberg, 1936.
Hollande, A. C.: Bull. d'histol. appliq. à la physiol., **1**:422, 1924.
Lazarow, A.; Cooperstein, S. J.; and Patterson, J. W.: Anat. Rec., **103**:482, 1949.
Lillie, R. S.: Am. J. Physiol., **7**:412, 1902.
Loele, W.: München. med. Wchnschr., **57**:2414, 1910.
Loele, W.: Zentralbl. f. allg. Path. u. path. Anat., **30**:614, 1920.
Marinesco, G.: Compt. rend. Soc. de biol., **82**:98, 1919.
Marinesco, G.: Bull. d'histol. appliq. à la physiol., **2**:239, 1925.
Mooc, F.: J. Cell. & Comp. Physiol., **22**:223, 1943.
Prenant, M.: Bull. d'histol. appliq. à la physiol., **1**:499, 1924.
Seabra, P.: M. Times, **74**:155, 1946.
Seabra, P.: J. Aviation Med., **18**:289, 1947.
Tsukamoto, H.: Hiroshima J. M. Sc., **1**:111, 1951.
Vernon, H. M.: Biochem. Ztschr., **47**:374, 1912.

Dopa Oxidase

Bittorf, A.: Arch. f. exper. Path. u. Pharmakol., **75**:143, 1913.
Bloch, B.: Arch. f. Dermat. u. Syph., **135**:77, 1921.
Bloch, B., and Schaaf, F.: Biochem. Ztschr., **162**:181, 1925.
Hasebroek, K.: Biol. Zentralbl., **41**:367, 1921.
Laidlaw, G. F.: Am. J. Path., **8**:477, 1932.
Laidlaw, G. F.: Anat. Rec., **53**:399, 1932.
Lison, L.: Compt. rend. Soc. de biol., **106**:41, 1927.
Miescher, G.: Arch. f. mikr. Anat., **97**:326, 1923.
Ohlmeyer, P., and Hüllstrung, H.: Ztschr. f. physiol. Chem., **282**:28, 1945.
Sato, K., and Brecher, L.: Arch. f. mikr. Anat., **104**:649, 1925.

Amine Oxidase

Bray, H. G.; James, S. P.; Raffen, I. M.; Ryman, B. E.; and Thorpe, W. V.: Biochem. J., **44**:618, 1949.
Epps, H. M. R.: Biochem. J., **39**:37, 1945.
Pugh, C. E. M., and Quastel, J. H.: Biochem. J., **31**:2307, 1937.

Peroxidases

properties of peroxidases

Battelli, F., and Stern, L.: Biochem. Ztschr., **13**:44, 1908.
Betke, K.: Klin. Wchnschr., **28**:788, 1950.
Chodat, R., and Bach, H.: Ber. d. deutsch. chem. Gesellsch., **36**:606, 1903.
Czyhlarz, E. von, and Fürth, O. von: Beitr. z. chem. Physiol. u. Path., **10**:358, 1907.
Dam, H., and Granados, H.: Acta physiol. Scandinav., **10**:162, 1945.
Madelung, W.: Ztschr. f. physiol. Chem., **71**:204, 1911.
Malowan, L. S.: Enzymologia, **7**:193, 1939.
Moore, B., and Whitney, E.: Biochem. J., **4**:136, 1909.
Prenant, M.: Arch. de morph. gén. et expér., **21**, 1924.
Theorell, H.: Adv. Enzymol., **7**:265, 1947.

technical

Beacom, D. N.: J. Lab. & Clin. Med., **11**:1092, 1926.
Dunn, R. C.: Stain Technol., **21**:65, 1946.
Duquénois, P., and Guth, T.: Compt. rend. Acad. sc., **233**:499, 1951.
Fiessinger, N.: Compt. rend. Soc. de biol., **82**:554, 1919.
Fischel, R.: München. med. Wchnschr., **57**:1203, 1910.
Fischel, R.: Wien. klin. Wchnschr., **23**:1567, 1910.
Fischel, R.: Arch. f. mikr. Anat., **33**:130, 1913.
García-Blanco, J.; Viña, J.; and Comin, J. L.: Rev. españ. fisiol., **4**:221, 1948.
Mancini, R. E.: Rev. Soc. argent. de biol., **16**:616, 1940.
Ralph, P. H.: Stain Technol., **16**:105, 1941.
Ritter, H. B., and Oleson, J. J.: Arch. Path., **43**:330, 1947.
Sato, A., and Sekiya, S.: Tohoku J. Exper. Med., **7**:111, 1926.
Sato, A., and Shoji, K.: J. Lab. & Clin. Med., **13**:1058, 1928.
Slonimski, P.: Compt. rend. Soc. de biol., **96**:1496, 1927.
Slonimski, P.: Arch. de biol., **42**:415, 1931.
Slonimski, P., and Lapinski, Z.: Ztschr. f. Zellforsch. u. mikr. Anat., **16**:653, 1932.
Suzuki, K.: Tohoku J. Exper. Med., **12**:235, 1928–29.
Villamil, M. F., and Mancini, R. E.: Rev. Soc. argent. de biol., **23**:215, 1947.

HISTOLOGICAL STUDIES

Dempsey, E. W.: Endocrinology, **34**:27, 1944.

Ferrari, R.: Boll. Soc. med.-chir. Pavia, **43**:585, 1929.

Mancini, R. E., and Villamil, M. F.: J. Nat. Cancer Inst., **10**:1379, 1950.

Prenant, M.: Compt. rend. Soc. de biol., **85**:808, 1921.

Prenant, M.: *Ibid.*, **85**:912, 1921.

Robertis, E. de, and Grasso, R.: Endocrinology, **38**:137, 1946.

Villamil, M. F., and Mancini, R. E.: Rev. Soc. argent. de biol., **23**:219, 1947.

Alkaline Phosphatase

A. TECHNICAL

Barger, J. D.: Arch. Path., **43**:620, 1947.

Cleland, K. W.: Proc. Linnean Soc. New South Wales, **75**:54, 1950.

Doyle, W. L.: Anat. Rec., **99**:633, 1947.

Doyle, W. L.: Quantitative aspects of the histochemistry of phosphatases. In Symposium on cytology. East Lansing: Michigan State College Press, 1951.

Doyle, W. L.; Omoto, J. H.; and Doyle, M. E.: Exper. Cell Research, **2**:20, 1951.

Eggers Lura, H., and Zander, H. A.: J. Dent. Research, **26**:473, 1947.

Feigin, I.; Wolf, A.; and Kabat, E. A.: Am. J. Path., **26**:647, 1950.

Holter, H.; Løvtrup, S.; and Rubin, I.: Acta chem. Scandinav., **5**:194, 1951.

Jacoby, F., and Martin, B. F.: Nature, **163**:875, 1949.

Martin, B. F.: Stain Technol., **24**:215, 1949.

Martin, B. F., and Jacoby, F.: J. Anat., **83**:351, 1949.

Mowry, R. W.: Bull. Internat. A. M. Mus., **30**:95, 1949.

Mutolo, V., and LiVoti, P.: Sicilia med., **6**:427, 1949.

Omoto, J. H.: Quantitative studies on the Gomori alkaline phosphatase staining in the rabbit kidney. Thesis, University of Chicago, 1951.

Takamatsu, H., and Sho, E.: Tr. Soc. Path. Jap., **32**:90, 1942.

Wang, K. J., and Grossman, M. I.: Anat. Rec., **104**:79, 1949.

Yokoyama, H. O.; Stowell, R. E.; and Mathews, R. M.: Anat. Rec., **109**:139, 1951.

Yokoyama, H. O.; Stowell, R. E.; and Tsuboi, K. K.: J. Nat. Cancer Inst., **10**:1367, 1950.

B. THE DISTRIBUTION OF ALKALINE PHOSPHATASE IN VARIOUS TISSUES AND UNDER VARIOUS CONDITIONS

a) Viruses, Bacteria, Plants

Bayliss, M.; Glick, D.; and Siem, R. A.: J. Bact., **55**:307, 1948.
Bray, J., and King, E. J.: J. Path. & Bact., **54**:287, 1942.
Glick, D., and Fischer, E. E.: Arch. Biochem., **8**:91, 1945.
Glick, D., and Fischer, E. E.: *Ibid.*, **11**:65, 1946.
Hilbe, J. J., and Marron, T. U.: Proc. Iowa Acad. Sc., **17**:235, 1940.
Wilmer, H. A.: Proc. Soc. Exper. Biol. & Med., **55**:206, 1944.
Yin, H. C.: New Phytologist, **44**:191, 1945.

b) Tissue Cultures; Nuclei

Baud, C. A., and Fulleringer, A.: Compt. rend. Acad. sc., **227**:645, 1948.
Biesele, J. J.: Proc. Nat. Cancer Conf., 1949, p. 34.
Biesele, J. J., and Wilson, A. Y.: Cancer Research, **11**:174, 1951.
Brachet, J.: Experientia, **2**:143, 1946.
Brachet, J., and Jeener, R.: Compt. rend. Soc. de biol., **140**:1121, 1946.
Brachet, J., and Jeener, R.: Biophys. et biochim. acta, **2**:423, 1948.
Chèvremont, M., and Firket, H.: Arch. de biol., **40**:441, 1949.
Chèvremont, M., and Firket, H.: Compt. rend. Soc. de biol., **143**:731, 1949.
Danielli, J. F.: XIII^{e} Congrès internat. de zoologie, Paris, 1949, p. 205.
Danielli, J. F., and Catcheside, D. G.: Nature, **156**:294, 1945.
Fajer, A.: Rend. Accad. naz. Lincei, ser. VIII, **7**:354, 1949.
Levi, G., and Fajer, A.: Rend. Accad. naz. Lincei, ser. VIII, **8**:98, 1950.
Willmer, E. N.: J. Exper. Biol., **19**:11, 1942.

c) Invertebrates

Anderson, J. M.: Physiol. Zoöl., **23**:308, 1950.
Bradfield, J. R. G.: Exper. Cell Research Suppl. I, 338, 1949.
Bullock, W. L.: Anat. Rec., **101**:688, 1948.
Day, M. F.: Australian J. Sc. Research, ser. B, Biol. Sc., **2**:31, 1949.
Kugler, O. E., and Birkner, M. L.: Physiol. Zoöl., **21**:105, 1948.
Montalenti, G., and de Nicola, M.: Experientia, **4**:314, 1948.
Montalenti, G., and de Nicola, M.: *Ibid.*, **4**:315, 1948.
Vitagliano, G., and de Nicola, M.: Nature, **162**:965, 1948.
Wicklund, E.: Nature, **161**:556, 1948.

d) Fish, Reptiles, Amphibians, Birds

Al-Hussaini, A. H.: Nature, **161**:274, 1948.
Al-Hussaini, A. H.: Quart. J. Micr. Sc., **90**:323, 1949.
Andrus, M., and Zarrow, M. X.: Proc. Soc. Exper. Biol. & Med., **72**:714, 1949.
Battaglia, B.: Pubbl. staz. zool. Napoli, **22**:79, 1949.
Berg, G. G., and Karczmar, A. G.: Anat. Rec., **106**:175, 1950.
Elftman, H., and Copenhaver, W. M.: Anat. Rec., **97**:385, 1947.
Jakowska, S.; Nigrelli, R. F.; and Gordon, M.: Cancer Research, **11**:259, 1951.
Ju, Fungchun: Tr. Soc. Path. Jap., **32**:79, 1942.
Junqueira, L. C. U.: J. Anat., **84**:369, 1950.
Karczmar, A. G., and Berg, G. G.: Anat. Rec., **106**:276, 1950.
King, T. J., and Nigrelli, R. F.: Proc. Soc. Exper. Biol. & Med., **72**:373, 1949.
Lorch, I. J.: Quart. J. Micr. Sc., **90**:381, 1949.
Moog, F.: Proc. Nat. Acad. Sc., **29**:176, 1943.
Moyson, F.: Ann. Soc. roy. zool. Belgique, **77**:68, 1947.
Osawa, S.: Embryologia, **2**:1, 1951.
Pettengill, O., and Copeland, D. E.: J. Exper. Zoöl., **108**:235, 1948.
Scharrer, E., and Sinden, J. A.: J. Comp. Neurol., **91**:331, 1949.
Seaman, A.: Anat. Rec., **103**:504, 1949.
Sinden, J. A., and Scharrer, E.: Proc. Soc. Exper. Biol. & Med., **72**:60, 1949.

e) Mammalians, Man

(Other species may be included under individual tissues)

DISTRIBUTION OF ENZYME UNDER NORMAL CONDITIONS

General

Bourne, G.: Quart. J. Exper. Physiol., **32**:1, 1943.
Gomori, G.: J. Cell. & Comp. Physiol., **17**:71, 1941.
McManus, J. F. A.: Bull. Internat. A. M. Mus., **28**:73, 1948.
Mawatari, H., and Ju, F.: Tr. Soc. Path. Jap., **32**:77, 1942.
Takamatsu, H.; Furuichi, T.; and Motoike, P.: Tr. Soc. Path. Jap., **30**:120, 1940.

Developmental

Furuichi, T., and Takamatsu, H.: Tr. Soc. Path. Jap., **31**:40, 1941.
Rossi, F.; Pescetto, G.; and Reale, E.: Ztschr. f. Anat. u. Entwcklngsgesch., **115**:500, 1951.

Muscle, Cartilage, Bone; Calcification

Bevelander, G., and Johnson, P. L.: Anat. Rec., **108**:1, 1950.
Bourne, G. H.: J. Anat., **83**:61, 1949.
Cappelin, M.: Atti Soc. med.-chir. Padova, **25**:86, 1947.
Cobb, J.: Anat. Rec., **103**:531, 1949.
Dempsey, E. W.; Wislocki, G. B.; and Singer, M.: Anat. Rec., **96**:221, 1946.
Drummond, D. H.; Tollman, J. P.; and Bisgard, J. D.: Arch. Surg., **40**:49, 1940.
Fell, H. B., and Robison, R.: Biochem. J., **23**:767, 1929.
Fell, H. B., and Robison, R.: *Ibid.*, **28**:2243, 1934.
Follis, R. H., Jr.: Bull. Johns Hopkins Hosp., **85**:360, 1949.
Follis, R. H., Jr.: Fed. Proc., **9**:330, 1950.
Freeman, S., and McLean, F. C.: Arch. Path., **32**:387, 1941.
Gomori, G.: Am. J. Path., **19**:197, 1943.
Greep, R. O.: Anat. Rec., **100**:667, 1948.
Kodama, S., and Takamatsu, H.: Tr. Soc. Path. Jap., **31**:30, 1941.
LiVoti, P.: Gior. ital. chir., **5**:478, 1949.
Lorch, I. J.: Quart. J. Micr. Sc., **88**:367, 1947.
Lorch, I. J.: *Ibid.*, **90**:183, 1949.
Lorch, I. J.: *Ibid.*, **90**:381, 1949.
Manigault, P.: Ann. Inst. océanog., **18**:331, 1939.
Montagna, W.: Anat. Rec., **103**:77, 1949.
Regen, E. M., and Wilkins, W. E.: J. Lab. & Clin. Med., **20**:250, 1934.
Ross, B. D.: Proc. Soc. Exper. Biol. & Med., **45**:531, 1940.
Soulairac, A., and Desclaux, P.: Compt. rend. Soc. de biol., **143**:470, 1949.
Tollman, J. P.; Drummond, D. H.; and McIntyre, A. R.: Arch. Surg., **40**:43, 1940.
Waldman, J.: Proc. Soc. Exper. Biol. & Med., **69**:262, 1948.
Wislocki, G. B.; Weatherford, H. L.; and Singer, M.: Anat. Rec., **99**:265, 1947.
Zorzoli, A.: Anat. Rec., **102**:445, 1948.

Teeth

Bevelander, G., and Johnson, P. L.: J. Cell. & Comp. Physiol., **26**:25, 1945.
Bruckner, R. J.: J. Dent. Research, **28**:55, 1949.
Engel, M. B., and Furuta, W.: Proc. Soc. Exper. Biol. & Med., **50**:5, 1942.

Greep, R. O.: J. Nat. Cancer Inst., **10**:1373, 1950.
Harris, E. S.: Anat. Rec., **107**:105, 1950.
Horowitz, N. H.: J. Dent. Research, **21**:519, 1942.
Morse, A., and Greep, R. O.: Anat. Rec., **97**:357, 1947.
Morse, A., and Greep, R. O.: *Ibid.*, **99**:379, 1947.
Verne, J.: J. dent. belge, **40**:135, 1949.
Verne, J., and Hébert, S.: Compt. rend. Soc. de biol., **142**:1392, 1948.
Verne, J.; Hébert, S.; and Charpal, O. de: Bull. d'histol. appliq. à la physiol., **26**:170, 1949.
Zander, H. A.: J. Dent. Research, **20**:347, 1941.

Skin and Its Appendages

Bunting, H.: Anat. Rec., **101**:5, 1948.
Bunting, H.; Wislocki, G. B.; and Dempsey, E. W.: Anat. Rec., **100**:61, 1948.
Fisher, I., and Glick, D.: Proc. Soc. Exper. Biol. & Med., **66**:14, 1947.
Johnson, P. L., and Bevelander, G.: Anat. Rec., **98**:147, 1947.
Johnson, P. L., and Bevelander, G.: *Ibid.*, **99**:633, 1947.
Johnson, P. L.; Butcher, E. O.; and Bevelander, G.: Anat. Rec., **93**:355, 1945.
Montagna, W.: Anat. Rec., **97**:356, 1947.
Montagna, W., and Hamilton, J. B.: Anat. Rec., **99**:550, 1947.
Montagna, W., and Hamilton, J. B.: Fed. Proc., **6**:166, 1947.
Montagna, W., and Noback, C. R.: Anat. Rec., **96**:111, 1946.
Montagna, W., and Noback, C. R.: Am. J. Anat., **81**:39, 1947.
Montagna, W.; Noback, C. R.; and Zak, F. G.: Am. J. Anat., **83**:409, 1948.
Montagna, W., and Parks, H. F.: Anat. Rec., **100**:297, 1948.

Nervous System; Sense Organs

Bourne, G. H.: Nature, **161**:445, 1948.
El-Baradi, A. F., and Bourne, G. H.: Science, **113**:660, 1951.
Hard, W. L.: Anat. Rec., **103**:465, 1949.
Hard, W. L., and Hawkins, R. K.: Anat. Rec., **108**:577, 1950.
Marchant, J.: J. Anat., **83**:227, 1949.
Schiebler, T. H.: Acta anat., **13**:233, 1951.
Shimizu, N.: J. Comp. Neurol., **93**:201, 1950.
Sinden, J. A., and Scharrer, E.: Proc. Soc. Exper. Biol. & Med., **72**:60, 1949.
Soulairac, A.; Desclaux, P.; Teysseyre, J.; and Chaneac, H.: Compt. rend. A. anat., XXXVII^{e} Réunion, Louvain, 1950, p. 471.

Sulkin, N. M.: Anat. Rec., **106**:252, 1950.
Sulkin, N. M., and Kuntz, A.: Anat. Rec., **108**:255, 1950.
Taylor, A. C.; Goldsmith, E. D.; and Bevelander, G.: Anat. Rec., **99**:634, 1947.
Wislocki, G. B., and Dempsey, E. W.: J. Comp. Neurol., **88**:319, 1948.

Blood; Hemopoietic System

Dalgaard, E., and Dalgaard, J. B.: Ugesk. f. laeger, **110**:513, 1948.
Kent, J. F.: Anat. Rec., **103**:474, 1949.
Noback, C. B., and Montagna, W.: Anat. Rec., **96**:279, 1946.
Paff, G. H.; Montagna, W.; and Bloom, F.: Cancer Research, **7**:789, 1947.
Rheingold, J. J., and Wislocki, G. B.: Blood, **3**:641, 1948.
Riley, J. F., and Drennan, J. M.: J. Path. & Bact., **61**:245, 1949.
Wachstein, M.: J. Lab. & Clin. Med., **31**:1, 1946.
Wislocki, G. B.; Bunting, H.; and Dempsey, E. W.: Anat. Rec., **98**:527, 1947.
Wislocki, G. B., and Dempsey, E. W.: Anat. Rec., **96**:249, 1946.

Pituitary, Thyroid

Abolinš, L.: Nature, **161**:556, 1948.
Dempsey, E. W.: Ann. New York Acad. Sc., **50**:336, 1949.

Reproductive Tract, Placenta, Etc.

Atkinson, W. B., and Gusberg, S. B.: Cancer, **1**:248, 1948.
Bern, H. A.: Anat. Rec., **104**:361, 1949.
Corner, G. W.: Science, **100**:270, 1944.
Corner, G. W.: Contrib. Embryol., **32**:1, 1948.
Dempsey, E. W., and Wislocki, G. B.: Am. J. Anat., **76**:277, 1945.
Hall, J. E.: Am. J. Obst. & Gynec., **60**:212, 1950.
Hard, W. L.: Am. J. Anat., **78**:47, 1946.
Krugelis, E. J.: J. Cell. & Comp. Physiol., **19**:376, 1942.
Mancini, R. E., and Burges, M. H.: J. Nat. Cancer Inst., **10**:1377, 1950.
Ober, K. G.: Klin. Wchnschr., **28**:9, 1950.
Ober, K. G., and Weber, M.: Klin. Wchnschr., **29**:53, 1951.
Pritchard, J. J.: J. Anat., **81**:352, 1947.
Pritchard, J. J.: *Ibid.*, **83**:10, 1949.
Pritchard, J. J.: *Ibid.*, **83**:71, 1949.
Ring, J. R.: Anat. Rec., **107**:121, 1950.
Soulairac, A., and Thibault, C.: Compt. rend. Acad. sc., **227**:446, 1948.

Talbert, G. B.; Stafford, R. A.; and Meyer, R. K.: Anat. Rec., **100**:718, 1948.
Tom, J. Y. S.: Anat. Rec., **103**:513, 1949.
Wimsatt, W. A.: Anat. Rec., **100**:724, 1948.
Wimsatt, W. A.: Am. J. Anat., **84**:63, 1949.
Wimsatt, W. A.: Anat. Rec., **103**:522, 1949.
Wimsatt, W. A.: *Ibid.*, **103**:564, 1949.
Wislocki, G. B.: Endocrinology, **44**:167, 1949.
Wislocki, G. B.: Anat. Rec., **106**:295, 1950.
Wislocki, G. B.; Deane, H. W.; and Dempsey, E. W.: Am. J. Anat., **78**:281, 1946.
Wislocki, G. B., and Dempsey, E. W.: Am. J. Anat., **77**:365, 1945.
Wislocki, G. B., and Dempsey, E. W.: Am. J. Anat., **78**:181, 1946.
Wislocki, G. B., and Dempsey, E. W.: Anat. Rec., **78**:1, 1946.
Wislocki, G. B., and Dempsey, E. W.: Endocrinology, **38**:90, 1946.
Wislocki, G. B., and Dempsey, E. W.: Am. J. Anat., **83**:1, 1948.
Wislocki, G. B., and Wimsatt, W. A.: Am. J. Anat., **81**:269, 1947.

Intestinal Tract

Deane, H. W., and Dempsey, E. W.: Anat. Rec., **93**:401, 1945.
Emmel, V. M.: Anat. Rec., **91**:39, 1945.
Hébert, S.: Arch. de biol., **61**:235, 1950.
Martin, B. F.: J. Anat., **85**:140, 1951.
Moog, F.: Anat. Rec., **108**:563, 1950.
Soulairac, A.: Compt. rend. Soc. de biol., **142**:645, 1948.
Voge, M., and Bern, H. A.: Anat. Rec., **104**:477, 1949.

Various Other Tissues

Antonini, F. M., and Weber, G.: Arch. "de Vecchi" per l'anat. pat. e med. clin., **16**:985, 1951.
Baxter, J. S.: J. Anat., **83**:82, 1949.
Cappelin, M.: Boll. Soc. ital. biol. sper., **23**:64, 1947.
Fawcett, D. W.: Anat. Rec., **103**:450, 1949.
Feyel-Cabanes, T.: Compt. rend. Soc. de biol., **143**:230, 1949.
Grossman, M. I.; Wang, C. C.; and Wang, K. J.: Proc. Soc. Exper. Biol. & Med., **78**:310, 1951.
Jacoby, F.: Nature, **158**:268, 1946.
Jacoby, F.: J. Physiol., **106**:33P, 1947.
Kritzler, R. A., and Gutman, A. B.: Am. J. Physiol., **134**:94, 1941.
Littrell, J. L.: Anat. Rec., **100**:691, 1948.
McAlpine, R. J.: Anat. Rec., **106**:220, 1950.
McAlpine, R. J.: *Ibid.*, **109**:189, 1951.
Noback, C. R.: Anat. Rec., **97**:359, 1947.

Ralph, P. H.: Anat. Rec., **103**:496, 1950.
Solymoss, B.: Kisérl. Orvostud., **2**:167, 1950.

Experimental and Pathological Conditions

effect of steroid hormones

Atkinson, W. B.: Anat. Rec., **100**:731, 1948.
Atkinson, W. B., and Elftman, H.: Proc. Soc. Exper. Biol. & Med., **62**:148, 1946.
Atkinson, W. B., and Elftman, H.: Endocrinology, **40**:30, 1947.
Atkinson, W. B., and Engle, E. T.: Endocrinology, **40**:327, 1947.
Atkinson, W. B., and Wright, P.: J. Nat. Cancer Inst., **10**:1372, 1950.
Baker, B. L., and Whitaker, W. L.: Endocrinology, **46**:544, 1950.
Bern, H. A.: Anat. Rec., **108**:524, 1950.
Bern, H. A.: Endocrinology, **48**:25, 1951.
Dempsey, E. W.; Greep, R. O.; and Deane, H. W.: Endocrinology, **44**:18, 1949.
Elftman, H.: Anat. Rec., **97**:331, 1947.
Elftman, H.: Endocrinology, **41**:85, 1947.
Grunt, J. A., and Leathem, J. H.: Proc. Soc. Exper. Biol. & Med., **72**:218, 1949.
Jeener, R.: Nature, **159**:578, 1947.
Jeener, R.: Biochem. et biophys. acta, **2**:439, 1948.
Kamell, S. A., and Atkinson, W. B.: Proc. Soc. Exper. Biol. & Med., **68**:537, 1948.
Kar, A. B.: Endocrinology, **46**:363, 1950.
Kochakian, C. D.: Am. J. Physiol., **152**:257, 1948.
Li, M. H., and Lu, Y. L.: Chinese J. Physiol., **17**:157, 1949.
Lu, Y. L.: Chinese J. Physiol., **17**:301, 1950.
Ludwig, A. W., and Boas, N. F.: Endocrinology, **46**:291, 1950.
Nataf, B., and Marois, M.: Compt. rend. Soc. de biol., **143**:1588, 1949.
Ring, J. R.: Anat. Rec., **103**:498, 1949.
Ring, J. R., and Levy, B. M.: J. Dent. Research, **28**:659, 1949.
Soulairac, A.; Desclaux, P.; and Tèysseyre, J.: Ann. d'endocrinol., **10**:535, 1949.
Soulairac, A.; Desclaux, P.; and Teysseyre, J.: Compt. rend. A. anat., **36**:638, 1949.
Soulairac, A., and Thibault, C.: Compt. rend. Soc. de biol., **142**: 1512, 1948.
Soulairac, A., and Thibault, C.: Compt. rend. A. anat., **35**:634, 1949.

Talmage, R. V.: Proc. Soc. Exper. Biol. & Med., **70**:719, 1949.
Thibault, C., and Soulairac, A.: Ann. d'endocrinol., **9**:555, 1948.
Tissières, A.: Acta anat., **5**:224, 1948.
Tissières, A.: *Ibid.*, **5**:235, 1948.
Verne, J., and Hébert, S.: Compt. rend. Soc. de biol., **142**:300, 1948.
Verne, J., and Hébert, S.: *Ibid.*, **143**:201, 1949.

RADIATION EFFECTS

Arvy, L.; Briffard, J. A.; and Gabe, M.: Compt. rend. Soc. de biol., **143**:233, 1949.
Doyle, W. L.: Am. J. Anat., **87**:79, 1950.
Petrakis, N. L.; Ashler, F. M.; and Ferkel, R. L.: Naval Radiol. Defense Lab. Rep., AD-126(B), June 2, 1949.
Ross, M. H., and Ely, J. O.: Am. J. Roentgenol., **62**:723, 1949.

VARIOUS OTHER EXPERIMENTAL CONDITIONS

Arvy, L., and Gabe, M.: Compt. rend. Soc. de biol., **143**:473, 1949.
Biesele, J. J., and Biesele, M. M.: Cancer Research, **4**:751, 1944.
Bunting, H., and White, R. F.: Arch. Path., **49**:590, 1950.
Danielli, J. F.; Fell, H. B.. and Kodicek, E.: Brit. J. Exper. Path., **26**:367, 1945.
Fell, H. B., and Danielli, J. F.: Brit. J. Exper. Path., **24**:196, 1943.
Herlant, M., and Timiras, P. S.: Endocrinology, **46**:243, 1950.
Kausche, G. A.; Landschütz, C.; and Sauthoff, R.: Ztschr. f. Naturforsch., **6b**:445, 1951.
Kodama, S., and Takamatsu, H.: Tr. Soc. Path. Jap., **30**:126, 1940.
Kroon, D. B.: Acta endocrinol., **2**:227, 1949.
Nákó, A., and Solymoss, B.: Orvosi Hetil., **92**:793, 1951.
Robertson, W. V. B.; Dunihue, F. W.; and Novikoff, A. B.: Brit. J. Exper. Path., **31**:545, 1950.
Shimizu, N.; Handa, Y.; Handa, J.; and Kumamoto, T.: Proc. Soc. Exper. Biol. & Med., **75**:696, 1951.
Steger, K.: Acta anat., **11**:245, 1950.
Sulkin, N. M.: Anat. Rec., **103**:510, 1949.
Zorzoli, A., and Nadel, E. M.: J. Nat. Cancer Inst., **10**:1366, 1950.

RENAL PATHOLOGY

Breedis, C.; Flory, C. M.; and Furth, J.: Arch. Path., **36**:402, 1943.
Gabe, M.: Compt. rend. Soc. de biol., **142**:1235, 1948.
Hepler, O. E.; Simonds, J. P.; and Gurley, H.: Proc. Soc. Exper. Biol. & Med., **44**:221, 1940.
McManus, J. F. A.: Medical diseases of the kidney. Philadelphia: Lea & Febiger, 1950.

McManus, J. F. A., and Mowry, R. W.: Bull. Internat. A. M. Mus., **28**:80, 1948.
Mancini, R. E.: J. Nat. Cancer Inst., **10**:1376, 1950.
Marsh, J. B., and Drabkin, D. L.: J. Biol. Chem., **168**:61, 1947.
Menten, M. L., and Janouch, M.: Proc. Soc. Exper. Biol. & Med., **63**:33, 1946.
Mowry, R. W.: J. Nat. Cancer Inst., **10**:1373, 1950.
Russell, W. O.; Rouse, E. T.; and Reed, J. A.: Arch. Path., **38**:40, 1944.
Soulairac, A. Compt. rend. Soc. de biol., **142**:643, 1948.
Soulairac, A.: *Ibid.*, **142**:778, 1948.
Soulairac, A.: J. de physiol., **41**:272A, 1949.
Soulairac, A.; Desclaux, P.; and Teysseyre, J.: Compt. rend. Soc. de biol., **143**:1595, 1949.
Wilmer, H. A.: J. Exper. Med., **78**:225, 1943.
Wilmer, H. A.: Arch. Path., **37**:227, 1944.

HEPATIC PATHOLOGY

Cleveland, F. P.; Richfield, D. F.; Gall, E. A.; and Schiff, L.: Arch. Path., **49**:333, 1950.
Cleveland, F. P.; Richfield, D. F.; Gall, E. A.; and Schiff, L.: J. Nat. Cancer Inst., **10**:1379, 1950.
Getty, R.: Anat. Rec., **103**:455, 1949.
Hard, W. L.: Anat. Rec., **103**:540, 1949.
Kritzler, R. A., and Beaubien, J.: Am. J. Path., **25**:1079, 1949.
Leathem, J. H.: Anat. Rec., **103**:549, 1949.
Mellors, R. C., and Sugiura, K.: Proc. Soc. Exper. Biol. & Med., **67**:242, 1948.
Sherlock, S., and Walshe, V.: J. Path. & Bact., **59**:615, 1947.
Stowell, R. E.; Lee, C. S.; and Tsuboi, K. K.: Am. J. Path., **26**:687, 1950.
Stowell, R. E.; Lee, C. S.; Tsuboi, K. K.; and Villasana, A.: Cancer Research, **11**:345, 1951.
Sulkin, N. M., and Gardner, J. H.: Anat. Rec., **100**:143, 1948.
Takamatsu, H., and Otsuki, T.: Gann, **35**:283, 1941.
Wachstein, M. Arch. Path., **40**:57, 1945.
Wachstein, M., and Zak, F. G.: Arch. Path., **42**:501, 1946.
Wachstein, M., and Zak, F. G.: Proc. Soc. Exper. Biol. & Med., **62**: 73, 1946.
Wachstein, M., and Zak, F. G.: Am. J. Clin. Path., **20**:99, 1950.

TUBERCULOSIS

Takeuchi, T., and Takamatsu, H.: Tr. Soc. Path. Jap., **29**:490, 1939.
Takeuchi, T., and Takamatsu, H.: *Ibid.*, **30**:127, 1940.

NEOPLASMS

Andres, A. G., and Perevoshchikova, K. A.: Ark. pat. anat. i pat. fisiol., **9**:35, 1947.

Atkinson, W. B., and Gusberg, S. B.: Cancer, **1**:248, 1948.

Kabat, E. A., and Furth, J.: Am. J. Path., **17**:303, 1941.

King, T. J., and Nigrelli, R. F.: Proc. Soc. Exper. Biol. & Med., **72**:373, 1949.

Landow, H.; Kabat, E. A.; and Newman, W.: Arch. Neurol. & Psychiat., **48**:518, 1942.

LiVoti, P., and Mutolo, V.: Sicilia med., **6**:373, 1949.

Paff, G. H.; Montagna, W.; and Bloom, F.: Cancer Research, **7**:798, 1947.

Pearson, B.; Novikoff, A. B.; and Morrione, T. G.: Cancer Research, **10**:557, 1950.

Solymoss, B.: Orvosi Hetil., **90**:125, 1949.

Takamatsu, H.: Gann, **33**:218, 1939.

Takamatsu, H.: *Ibid.*, **34**:81, 1940.

5-Nucleotidase

Antonini, F. M., and Weber, G.: Arch. "de Vecchi" per l'anat. pat. e med. clin., **16**:985, 1951.

Biesele, J. J.: Proc. Nat. Cancer Conf., 1949, p. 34.

Biesele, J. J., and Wilson, A. Y.: Cancer Research, **11**:174, 1951.

Heppel, L. A., and Hilmoe, R. J.: J. Biol. Chem., **188**:665, 1951.

Pearse, A. G. Everson: J. Clin. Path., **4**:1, 1951.

Acid Phosphotase

A. TECHNICAL

Moog, F.: J. Cell. & Comp. Physiol., **22**:95, 1943.

Smith, W. K.: Anat. Rec., **100**:776, 1948.

Smith, W. K.: *Ibid.*, **102**:523, 1948.

Sulkin, N. M., and Kuntz, A.: Anat. Rec., **99**:639, 1947.

Takeuchi, T., and Tanoue, M.: Igaku to Seibutzugaku, **19**:316, 1951.

B. DISTRIBUTION OF THE ENZYME IN VARIOUS TISSUES

a) Plants; Amebae

Carrera, G. M.: Proc. Soc. Exper. Biol. & Med., **73**:682, 1950.

Carrera, G. M., and Changus, G. W.: Proc. Soc. Exper. Biol. & Med., **68**:610, 1948.

Kugler, O. E., and Bennett, E. H.: Stain Technol., **22**:9, 1947.

b) Nervous System

Bodian, D., and Mellors, R. C.: Proc. Soc. Exper. Biol. & Med., **55**:243, 1944.
Bodian D., and Mellors, R. C.: J. Exper. Med., **81**:460, 1945.
Hard, W. L., and Ferrara, B.: Anat. Rec., **97**:390, 1947.
Hard, W. L., and Hawkins, R. K.: Anat. Rec., **108**:577, 1950.
Kuntz, A., and Sulkin, N. M.: Anat. Rec., **99**:634, 1947.
Lassek, A. M., and Bucker, E. D.: Anat. Rec., **97**:395, 1947.
Meath, J. A., and Pope, A.: Fed. Proc., **9**:204, 1950.
Shimizu, N.: J. Comp. Neurol., **43**:201, 1950.
Sinden, J. A., and Scharrer, E.: Proc. Soc. Exper. Biol. & Med., **72**:60, 1949.
Smith, W. K., and Luttrell, C.: Anat. Rec., **97**:426, 1947.

c) Blood and Hemopoietic System

Montagna, W., and Noback, C. R.: Science, **106**:19, 1947.
Montagna, W., and Noback, C. R.: Anat. Rec., **100**:535, 1948.
Noback, C. R., and Montagna, W.: Anat. Rec., **96**:279, 1946.
Rabinovitch, M., and Andreucci, D.: Blood, **4**:580, 1949.
Rabinovitch, M., and Junqueira, L. C. U.: Science, **170**:322, 1948.
Rheingold, J. J., and Wislocki, G. B.: Blood, **3**:641, 1948.

d) Female Reproductive Tract; Placenta

Wislocki, G. B., and Dempsey, E. W.: Endocrinology, **38**:90, 1946.
Wislocki, G. B., and Dempsey, E. W.: Am. J. Anat., **83**:1, 1948.
Wislocki, G. B.; Deane, H. W.; and Dempsey, E. W.: Am. J. Anat., **78**:281, 1946.

e) Various Other Tissues

Deane, H. W., and Dempsey, E. W.: Anat. Rec., **93**:401, 1945.
Dempsey, E.: Ann. New York Acad. Sc., **50**:336, 1949.
Fisher, I., and Glick, D.: Proc. Soc. Exper. Biol. & Med., **66**:14, 1947.
Getty, R.: Anat. Rec., **103**:455, 1949.
Huggins, C.; Stevens, R. E.; and Hodges, C. V.: Arch. Surg., **43**:209, 1941.
Junqueira, L. C. U.: J. Anat., **84**:369, 1950.
Junqueira, L. C. U.; Rabinovitch, M.; and Fajer, A.: Anat. Rec., **100**:682, 1948.
Montagna, W., and Hamilton, J. B.: Anat. Rec., **99**:560, 1947.
Montagna, W., and Hamilton, J. B.: Fed. Proc., **6**:166, 1947.

Montagna, W., and Noback, C. R.: Anat. Rec., **96**:111, 1946.
Montagna, W., and Noback, C. R.: Am. J. Anat., **81**:39, 1947.
Montagna, W., and Parks, H. F.: Anat. Rec., **100**:297, 1948.
Noback, C. R., and Paff, G. H.: Anat. Rec., **103**:552, 1949.
Palade, G. E.: Anat. Rec. **106**:310, 1950.
Seaman, A.: Anat. Rec., **103**:504, 1949.
Soulairac, A., and Desclaux, P.: Compt. rend. Soc. de biol., **143**:470, 1949.

Phosphamidase

Holter, H., and Si-Oh-Li: Compt. rend. trav. lab. Carlsberg, sér. chim., **27**:393, 1951.
Scharrer, E., and Sinden, J. A.: J. Comp. Neurol., **91**:331, 1949.
Sinden, J. A., and Scharrer, E.: Proc. Soc. Exper. Biol. & Med., **72**:60, 1949.

Esterases

general

Gomori, G.: Arch. Path., **41**:121, 1946.
Nishiyama, Y.: Igaku to Seibutzugaku, **17**:217, 1950.
Sneath, P. H. A.: Nature, **166**:699, 1950.

various normal and nonneoplastic tissues

Baradi, A. F., and Bourne, G. H.: Nature, **168**:977, 1951.
Bullock, W. L.: J. Morphol., **81**:185, 1949.
Cammermeyer, J.: J. Comp. Neurol., **90**:121, 1949.
Fawcett, D. W.: Science, **105**:123, 1947.
Hunter, R. L.: Proc. Soc. Exper. Biol. & Med., **78**:56, 1951.
Quen, M.: Anat. Rec., **106**:281, 1950.
Saka, O.: 5e Cong. internat. de path. comp. Istanbul, 1949, p. 484.
Stowell, R. E., and Lee, C. S.: Arch. Path., **50**:519, 1950.
Vogel, F. S.: J. Exper. Med., **93**:305, 1951.
Vogel, S.: Am. J. Path., **26**:670, 1950.
Wachstein, M.: J. Exper. Med., **84**:25, 1946.

neoplastic conditions

Cohen, R. B.: Nachlas, M. M.; and Seligman, A. M.: Cancer Research, **11**:709, 1951.
Kung, S. K.: J. Nat. Cancer Inst., **9**:435, 1949.
Mark, D. D.: Arch. Path., **49**:545, 1950.
Menk, K. F., and Hyer, H.: Arch. Path., **48**:305, 1949.

CHOLINESTERASE

BERGNER, A. D., and DURLACHER, S. H.: Am. J. Path., **27**:1011, 1951.
HARD, W. L.: Anat. Rec., **106**:201, 1950.
HARD, W. L., and Fox, M. D.: Science, **112**:598, 1950.
HARD, W. L., and PETERSON, A. C.: Anat. Rec., **108**:57, 1950.
HARD, W. L., and PETERSON, A. C.: J. Neuropath. & Exper. Neurol., **10**:48, 1951.
KOELLE, G. B.; FRIEDENWALD, J. S.; KOELLE, E. S.; and WURZBACHER, W.: J. Nat. Cancer Inst., **10**:1364, 1950.
KOELLE, G. B.; KOELLE, E. S.; and FRIEDENWALD, J. S.: J. Pharmacol. & Exper. Therap., **100**:180, 1950.
SAWYER, H.; DAVENPORT, C.; and ALEXANDER, L. M.: Anat. Rec., **106**:287, 1950.
SCHARRER, E., and SINDEN, J. A.: J. Comp. Neurol., **91**:331, 1949.
SINDEN, J. A., and SCHARRER, E.: Proc. Soc. Exper. Biol. & Med., **72**:60, 1949.

INDEXES

AUTHOR INDEX

SUBJECT INDEX

[PRINTED IN U·S·A·]